



2017

The C-Rel Transcription Factor Controls Metabolism And Proliferation Of Human T Cells

George S. Luo

University of Pennsylvania, georgeluo@outlook.com

Follow this and additional works at: <https://repository.upenn.edu/edissertations>

 Part of the [Allergy and Immunology Commons](#), [Immunology and Infectious Disease Commons](#), and the [Medical Immunology Commons](#)

Recommended Citation

Luo, George S., "The C-Rel Transcription Factor Controls Metabolism And Proliferation Of Human T Cells" (2017). *Publicly Accessible Penn Dissertations*. 2450.
<https://repository.upenn.edu/edissertations/2450>

This paper is posted at ScholarlyCommons. <https://repository.upenn.edu/edissertations/2450>
For more information, please contact repository@pobox.upenn.edu.

The C-Rel Transcription Factor Controls Metabolism And Proliferation Of Human T Cells

Abstract

Environment-driven metabolic reprogramming is a persistent feature throughout the life cycle of T cells and is essential for their normal development and functioning. Consequently, abnormal metabolic adaptation underpins a number of human diseases, including cancer. Although the great metabolic adaptation potential helps T cells to survive nutrient-restricted regions in the periphery and fulfill their duty in immune surveillance and other functions, it also helps T cell derived tumor cells to survive the nutrient-deprived tumor microenvironment, thus making them more resilient and difficult to combat. It has long been shown that cancer cells exhibit a metabolic shift from oxidative phosphorylation to aerobic glycolysis, a phenomenon known as the Warburg effect. A similar metabolic shift is also observed in activated T cells, suggesting a common mechanism between immune activation and tumorigenesis. However, this mechanism remains to be elucidated. The lymphoid-specific NF- κ B family transcription factor c-Rel is a key regulator of B- and T-cell activation and immune response, and has been implicated in a number of hematopoietic cancers. To investigate the function and regulation of c-Rel in human T cells, we generated c-Rel knockout human T cell line Jurkat cells using the CRISPR/Cas9 system and analyzed the resulting phenotype. Our results revealed that c-Rel is a key metabolic regulator that facilitates T cell growth and proliferation by regulating energy metabolism to promote glycolysis and mitochondrial respiration. Disruption of c-Rel in Jurkat cells leads to dramatic reduction in cell growth and broad defects in both glycolysis and mitochondrial respiration. These metabolic defects are also observed in primary T cells isolated from c-Rel knockout mice, demonstrating c-Rel's critical role in regulating T cell metabolism across species. Moreover, c-Rel knockout (KO) cells failed to adapt to nutrient-limited conditions and lost their proliferative capacity. Mechanistically, c-Rel is upregulated in response to nutrient stress and drives the expression of c-Myc – a potentiator of many genes in the glycolytic and mitochondrial respiratory pathway. Overexpression of c-Myc in c-Rel KO cells compensated for the loss of c-Myc induction under nutrient stress, and reversed the proliferative and metabolic defects. These results establish that c-Rel is a key stress responder and metabolic regulator that maintains cell growth and survival under nutrient stress and promotes the Warburg effect. Thus, strategies targeting c-Rel may provide an effective way to suppress cancer metabolism.

Degree Type

Dissertation

Degree Name

Doctor of Philosophy (PhD)

Graduate Group

Biology

First Advisor

Youhai H. Chen

Keywords

c-Rel, glycolysis, mitochondrial respiration, NF- κ B, nutrient stress, T cell metabolism

Subject Categories

Allergy and Immunology | Immunology and Infectious Disease | Medical Immunology

**THE C-REL TRANSCRIPTION FACTOR CONTROLS METABOLISM AND
PROLIFERATION OF HUMAN T CELLS**

George S. Luo

A DISSERTATION

in

Biology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2017

Supervisor of Dissertation

Youhai H. Chen, M.D., Ph.D.
Professor of Pathology and Laboratory Medicine

Graduate Group Chairperson

Michael Lampson, Ph.D.
Associate Professor of Biology

Dissertation Committee

Wei Guo, Ph.D., Professor of Biology (Chair)

Erfei Bi, Ph.D., Professor of Cell and Developmental Biology

Wayne W. Hancock, M.D., Ph.D., Professor of Pathology and Laboratory Medicine

Robert P. Ricciardi, Ph.D., Professor of Microbiology

Andrew D. Wells, Ph.D., Associate Professor of Pathology and Laboratory Medicine

THE C-REL TRANSCRIPTION FACTOR CONTROLS METABOLISM AND
PROLIFERATION OF HUMAN T CELLS

COPYRIGHT

2017

George S. Luo

This work is licensed under the
Creative Commons Attribution-
NonCommercial-ShareAlike 3.0
License

To view a copy of this license, visit

<https://creativecommons.org/licenses/by-nc-sa/3.0/us/>

ACKNOWLEDGMENTS

I want to thank my mentor, Dr. Youhai Chen for providing me the wonderful opportunity to be part of his lab. His generous support and wise advice, combined with his programmatic approach to scientific challenges, demonstrated all the best qualities a scientist can have. In our conversations, he often presses me on the fine points of experimental design. Dr. Chen has inspired my desire to become a rigorous, methodologically sophisticated scholar, to which I am forever grateful. In addition, the warm and supportive environment in Dr. Chen's lab mirrored the happy years I spent at Caltech as an undergraduate, and his unwavering support and encouragement to my future endeavors helped me to embrace my aspirations and follow in his footsteps with great confidence.

I also want to thank my mentors at Caltech, Dr. David Baltimore and late Dr. Seymour Benzer. They both have encouraged me to set ambitious goals and stimulated my appreciation that there is more than one pathway. Dr. Baltimore modeled for me scientific courage, and his advocacy of science as an agent of social change has also affected me deeply. In Dr. Benzer, I have found a mentor with an unsurpassed zeal for science and scientific discovery. He is a great source of inspiration for me as I view him as someone motivated by genuine intellectual curiosity.

I want to thank Dr. Xinyuan Li for his intellectual and experimental contribution to this work. His expertise in metabolic analysis and data mining,

combined with rigorous yet expeditious experimental execution, played a key part in this project. I also want to thank Dr. Honghong Sun, who helped me on numerous projects and experiments throughout my time in the lab.

Finally, I want to thank members of my thesis committee: Drs. Wei Guo, Erfei Bi, Wayne Hancock, Robert Ricciardi, and Andrew Wells, for devoting their valuable time to provide critical opinions and guidance on my thesis work. Their advice and suggestions helped to address several key issues along the way.

ABSTRACT

THE C-REL TRANSCRIPTION FACTOR CONTROLS METABOLISM AND PROLIFERATION OF HUMAN T CELLS

George S. Luo

Youhai H. Chen

Environment-driven metabolic reprogramming is a persistent feature throughout the life cycle of T cells and is essential for their normal development and functioning. Consequently, abnormal metabolic adaptation underpins a number of human diseases, including cancer. Although the great metabolic adaptation potential helps T cells to survive nutrient-restricted regions in the periphery and fulfill their duty in immune surveillance and other functions, it also helps T cell derived tumor cells to survive the nutrient-deprived tumor microenvironment, thus making them more resilient and difficult to combat. It has long been shown that cancer cells exhibit a metabolic shift from oxidative phosphorylation to aerobic glycolysis, a phenomenon known as the Warburg effect. A similar metabolic shift is also observed in activated T cells, suggesting a common mechanism between immune activation and tumorigenesis. However, this mechanism remains to be elucidated. The lymphoid-specific NF- κ B family transcription factor c-Rel is a key regulator of B- and T-cell activation and immune response, and has been implicated in a number of hematopoietic cancers. To investigate the function and regulation of c-Rel in human T cells, we generated c-Rel knockout human T cell line Jurkat cells using the CRISPR/Cas9 system and analyzed the resulting phenotype. Our results revealed that

c-Rel is a key metabolic regulator that facilitates T cell growth and proliferation by regulating energy metabolism to promote glycolysis and mitochondrial respiration. Disruption of c-Rel in Jurkat cells leads to dramatic reduction in cell growth and broad defects in both glycolysis and mitochondrial respiration. These metabolic defects are also observed in primary T cells isolated from c-Rel knockout mice, demonstrating c-Rel's critical role in regulating T cell metabolism across species. Moreover, c-Rel knockout (KO) cells failed to adapt to nutrient-limited conditions and lost their proliferative capacity. Mechanistically, c-Rel is upregulated in response to nutrient stress and drives the expression of c-Myc – a potentiator of many genes in the glycolytic and mitochondrial respiratory pathway. Overexpression of c-Myc in c-Rel KO cells compensated for the loss of c-Myc induction under nutrient stress, and reversed the proliferative and metabolic defects. These results establish that c-Rel is a key stress responder and metabolic regulator that maintains cell growth and survival under nutrient stress and promotes the Warburg effect. Thus, strategies targeting c-Rel may provide an effective way to suppress cancer metabolism.

Table of Contents

ACKNOWLEDGMENTS	III
ABSTRACT	V
TABLE OF CONTENTS	VII
LIST OF FIGURES	IX
CHAPTER 1: INTRODUCTION	1
<i>Cellular energy metabolism in T cells.....</i>	<i>1</i>
<i>The Warburg effect.....</i>	<i>6</i>
<i>NF-κB transcription factors.....</i>	<i>7</i>
<i>NF-κB family member c-Rel.....</i>	<i>10</i>
<i>c-Rel in diseases</i>	<i>12</i>
CHAPTER 2: C-REL IS A KEY REGULATOR OF T CELL PROLIFERATION UNDER NUTRIENT STRESS	14
INTRODUCTION	14
RESULTS	16
<i>Generating c-Rel knockout (KO) Jurkat cells using the CRISPR/Cas9 system</i>	<i>16</i>
<i>c-Rel KO cells show impaired glucose and glutamine utilization.....</i>	<i>19</i>
<i>c-Rel is required for c-Myc induction upon PMA/ionomycin stimulation or serum starvation</i>	<i>20</i>
<i>The 2kb c-Myc promoter region is sufficient for response to c-Rel activation.....</i>	<i>21</i>
C-MYC REVERSES THE CELL GROWTH DEFECT OF C-REL KO CELLS	22
DISCUSSION.....	22
FIGURES.....	25
CHAPTER 3: C-REL REGULATES T CELL METABOLISM BY PROMOTING GLYCOLYSIS AND MITOCHONDRIAL RESPIRATION.....	43
INTRODUCTION	43
RESULTS	44
<i>c-Rel KO cells show reduced glucose consumption.....</i>	<i>44</i>
<i>Glycolysis and mitochondrial respiration are severely compromised in c-Rel knockout cells</i>	<i>44</i>
<i>c-Myc is partially responsible for the metabolic defects of c-Rel KO cells</i>	<i>46</i>
C-REL PROMOTES GLYCOLYSIS AND MITOCHONDRIAL RESPIRATION IN PRIMARY T CELLS IN MICE.....	46
DISCUSSION.....	47
FIGURES.....	50
CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS.....	55
DISCUSSION:	55
FUTURE DIRECTIONS:.....	58
CHAPTER 5: MATERIALS AND METHODS	61
CELL LINES.....	61
ANIMALS.....	61
PLASMID VECTORS.....	61
PLASMID CONSTRUCTION	62
LENTIVIRUS PRODUCTION AND CELL TRANSDUCTION	63

GENERATION OF C-REL KO CELLS USING THE CRISPR/CAS9 SYSTEM.....	64
CLONAL SELECTION TO ESTABLISH PURE C-REL KO LINES.	64
PMA/IONOMYCIN STIMULATION OF JURKAT CELLS.....	65
MOUSE T CELL ISOLATION AND STIMULATION	65
WESTERN BLOTTING	65
CELL SORTING BY FLOW CYTOMETRY	66
QUANTITATIVE RT-PCR.....	66
LUCIFERASE REPORTER ASSAY	67
SEAHORSE METABOLIC ANALYSIS	67
STATISTICAL ANALYSES	69
REFERENCES.....	71

LIST OF FIGURES

FIGURE 1 Design of a CRISPR/Cas9 system to create human c-Rel knockout cells...	25
FIGURE 2 c-Rel knockout Jurkat cells show reduced cell growth.....	26
FIGURE 3 c-Rel knockout Jurkat cells fail to express IL-2 upon PMA/ionomycin stimulation.....	27
FIGURE 4 c-Rel KO Jurkat cells show severe growth and survival defect under low serum concentrations.....	28
FIGURE 5 c-Rel is required for cell growth under serum starvation.....	29
FIGURE 6 Rescuing c-Rel expression in c-Rel KO cells using a lentiviral vector.....	30
FIGURE 7 Re-expressing c-Rel in c-Rel KO cells rescues their growth defect.....	31
FIGURE 8 c-Rel is required for glucose and glutamine utilization	32
FIGURE 9 Microarray analysis showing significant biological processes affected by c-Rel knockout	33
FIGURE 10 c-Rel knockout leads to downregulation of a number of metabolism related genes.....	34
FIGURE 11 c-Myc is identified as a c-Rel target through microarray analyses	35
FIGURE 12 c-Rel is required for c-Myc induction under PMA/ionomycin stimulation	36
FIGURE 13 c-Rel knockout leads to deficiency in c-Myc induction under serum starvation	37
FIGURE 14 Re-expressing c-Rel in c-Rel KO cells restores c-Myc induction under serum starvation	38
FIGURE 15. c-Rel drives c-Myc promoter activity.....	39
FIGURE 16 Rescue of c-Myc deficiency through expression of c-Myc.....	40
FIGURE 17 c-Myc expression compensates c-Myc induction failure in c-Rel KO cells	41
FIGURE 18 c-Myc rescues growth defect of c-Rel KO cells under serum starvation.	42
FIGURE 19 c-Rel KO cells show reduced glucose consumption	50
FIGURE 20 c-Rel KO Jurkat cells exhibit impaired glycolysis that can be partially reversed by c-Myc overexpression	51
FIGURE 21 c-Rel KO Jurkat cells show compromised mitochondrial respiration that can be rescued by c-Myc.....	52
FIGURE 22 c-Myc does not fully rescue GLUT1 deficiency in c-Rel KO cells	53
FIGURE 23 Primary CD4 ⁺ T cells isolated from c-Rel KO mice show significant defects in both mitochondrial respiration and glycolysis.....	54
FIGURE 24 Proposed mechanism for c-Rel-regulated metabolic reprogramming....	60
FIGURE 25 Process of generating c-Rel knockout Jurkat cells and control cells	70

CHAPTER 1: Introduction

Cellular energy metabolism in T cells

The human body is composed of plethora of cell types that require varying levels of energy supply to support their basic and functional activity. The versatility of many cell types in the immune system demands that they produce higher energy to support functions such as migration, antigen presentation, cytokine production and phagocytosis. Nevertheless, in all cell types, free energy is mainly presented in the form of adenosine 5'-triphosphate (ATP). ATP is produced most efficiently through the process of oxidative phosphorylation (OXPHOS) in the mitochondrial respiratory chain (Chaban et al., 2014). During this process, reduced forms of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH₂) are oxidized to generate a proton gradient, which in turn powers the phosphorylation of adenosine 5'-diphosphate (ADP) to produce ATP. NADH and FADH₂ can be derived from multiple sources, including glucose, the amino acid glutamine, and free fatty acids. The typical OXPHOS reaction produces approximately 30 molecules of ATP from one molecule of glucose via pyruvate intermediate. As its name implies, OXPHOS is strongly dependent on oxygen availability. In the absence of oxygen, a less efficient process, glycolysis, can be used to produce ATP from glucose. However, glycolysis has much lower glucose-to-ATP conversion efficiency, yielding only two molecules of ATP from each molecule of glucose. Despite the lower ATP-per-glucose

yield, glycolysis can produce ATP in greater amount at a faster rate than OXPHOS (Voet et al., 2011), making it more suitable for cancer and normal proliferating cells.

In addition to oxygen availability, the mode of ATP production chosen by cells also depends on their functional requirement. For example, different subsets of T cells with distinct functions adapt different energetic and biosynthetic metabolic programs. Conversely, changes in cell metabolism can enhance or suppress immune functions at both the cell and system levels (MacIver et al., 2013).

The activation of naïve CD4⁺ and CD8⁺ T cells upon antigen/ T-cell receptor (TCR) stimulation leads to differentiation into various types of effector and memory cells, which are capable of migrating into affected tissues and secrete effector cytokines (Geginat et al., 2013). Being quiescent and non-proliferating cells, naïve T cells generate energy mainly through mitochondria-dependent OXPHOS to support basic housekeeping tasks such as vesicle transport, cytoskeleton and membrane maintenance, and proteasomal recycling (Guppy et al., 1993). In contrast, activated T cells need a metabolic program that can support the increased energy demands associated with rapid cell growth, proliferation, and effector functions such as cytotoxicity and cytokine production. In addition, the activation-induced differentiation, clonal expansion and cytokine production requires processing of metabolites into building materials to support these activities (Verbist et al., 2012). Therefore, even in the presence of sufficient oxygen, activated T cells still use glycolysis, i.e., aerobic glycolysis, as their primary mode of energy metabolism (Fox

et al., 2005). Activated T cells also show a substantial increase in glucose uptake through the anabolic pentose phosphate pathway, leading to increased synthesis of nucleotides, proteins, and lipids (Maciolek et al., 2014). The conversion of effector T cells into quiescent memory T cells is accompanied by yet another metabolic switch back to OXPHOS and fatty acid catabolism (Geginat et al., 2001), demonstrating the plasticity of T cells in metabolic reprogramming. In term of CD4 + T helper (Th) cells, although Th1, Th2, and Th17 cells all rely on glucose for ATP production, Th17 cells have higher rates of glycolysis (Chang et al., 2013). In contrast to these pro-inflammatory T helper cells, the anti-inflammatory regulatory T (Treg) cells have increased OXPHOS – a key metabolic difference between the reciprocal Th17 and Treg cells (Michalek et al., 2011; Shi et al., 2011).

The metabolic switch from glucose and fatty acid oxidation through the TCA cycle to the glycolytic, glutaminolytic, and pentose-phosphate pathways following T cell activation has several known regulators. One of them is the transcription factor v-Myc myelocytomatosis viral oncogene homolog (c-Myc). Upon activation through TCR, c-Myc is significantly upregulated at both the mRNA and the protein levels and promotes the expression of an assortment of genes that are central to glycolysis and glutaminolysis (Wang et al., 2011). These include the glucose transporter GLUT1, hexokinase 2 (HK2), phosphofructokinase (PFKM), enolase 1 (ENO1), lactate dehydrogenase A (LDHA), and pyruvate dehydrogenase kinase PDK1 (Kim et al., 2004 and 2007; Osthus et al., 2000; Shim et al., 1997). The latter two convert pyruvate to

lactate during glycolysis, and block the entry of pyruvate into the TCA cycle, respectively.

Activation through TCR, costimulatory molecules and cytokine receptors also leads to upregulation of the phosphatidylinositol 3-kinase (PI3K), Protein kinase B (AKT), and mechanistic-mammalian target of rapamycin (mTOR) pathway, which plays a crucial role in promoting glucose metabolism and aerobic glycolysis, and preventing T cell anergy (Frauwirth et al., 2002; van der Windt et al., 2012). Activated Akt and mTOR complex 1 (mTORC1) promote plasma membrane translocation of GLUT1 and inhibit its internalization (Gerriets et al., 2012). They also promote expression of amino acid transporter and activation of glycolytic enzymes to increase glucose and glutamine flux (Edinger, 2007; John et al., 2011). Furthermore, mTOR can enhance protein translation by phosphorylating eukaryotic translation initiation factor 4E-binding protein 1 (4EBP) (Tandon et al., 2011).

In contrast to mTOR that promotes anabolic processes to support cell growth, the AMP activated protein kinase (AMPK) is a metabolic regulator that senses increased ratio of AMP to ATP and upregulates energy-producing catabolic pathways to maximize ATP production (Hardie, 2007), while at the same time suppressing ATP consumption through inhibition of mTOR. Immunosuppressive Treg cells show increased OXPHOS that corresponds to increased phosphorylation of AMPK (Michalek et al., 2011; Cobbold, 2013).

Another known metabolic regulator is hypoxia-inducible factor-1 (HIF-1). Hypoxia, i.e., abnormally low oxygen concentration ($\leq 1\% \text{ O}_2$) due to insufficient oxygen supply and/or increased oxygen consumption, can occur under a number of pathophysiological conditions such as inflammation and tumor growth (Caldwell et al., 2001; Gaber et al., 2005). The functions of immune cells demand that they have the ability to travel to these sites, adapt to the local environment and perform their intended functions. Because most T cell functions are highly energy-dependent, they need to be able to quickly switch from OXPHOS and aerobic glycolysis to anaerobic glycolysis in response to hypoxia (Semenza, 2012). HIF-1 is a transcription factor that serves as an oxygen sensor and an effector of hypoxic response (Semenza, 2007 and 2014). Upon hypoxia, the α -subunit HIF-1 is stabilized and enters the nucleus to dimerize with the β -subunit, resulting in activation. The activated HIF-1 then drives transcription of a large number of targets, including angiogenic factors such as VEGF, chemokine receptor CXCR4, as well as a number of glycolysis-related genes (Schodel et al., 2011). HIF-1 has been shown to promote Treg functions by suppressing T cell activation and effector functions, thus providing a link between hypoxia and immunosuppression (Ben-Shoshan et al., 2008; Ohta et al., 2011; McNamee et al., 2013; Bollinger et al., 2014). However, HIF-1 has also been shown to bias naïve CD4^+ T-cell differentiation towards the Th17 lineage through upregulation of ROR γ t and downregulation of Foxp3, thereby blocking the generation of Treg cells (Korn et al., 2009; Dang et al., 2011). Therefore, HIF-1 provides a good example of how

environmental stress drives metabolic change, which in turns alters T cell differentiation and function (Ikejiri et al., 2012).

Metabolic alteration is also implicated in failed immune responses. For example, TCR stimulation with incomplete CD28 co-stimulation leads to failure to switch from OXPHOS to glycolysis, causing deficiency in nutrient supply and inability to activate, grow, and proliferate (Powell et al., 2010; Du et al., 2012). This state of anergy can be induced by tumors for immune evasion. Tumor-associated antigen presenting cells (APCs) has been shown to alter expression of B7 family membrane proteins, thereby disrupting CD28 signaling (Crespo et al., 2013). In addition, hypoxia in the tumor microenvironment can promote immunosuppressive functions of Treg cells in a HIF-1–dependent manner (Ohta et al., 2006 and 2012). Nevertheless, recent advances in checkpoint blockade cancer immunotherapy targeting immunoinhibitory receptors CTLA4 and PD-1 have demonstrated that T cells are fundamentally capable of executing effector functions in the nutrient-deprived tumor microenvironment (Pardoll, 2012; Pico de Coaña et al., 2015).

The Warburg effect

The switch from oxidative phosphorylation to aerobic glycolysis is not unique to activated T cells. Indeed, this phenomenon was first observed nearly a century ago, when Otto Warburg discovered that cancer cells shift their energy supply from mitochondrial respiration to glycolysis, even in the presence of sufficient oxygen that

could support OXPHOS (Warburg, 1956). The aerobic glycolysis process produces ATP and lactate in the cytoplasm without the need of oxygen. This metabolic reprogramming was later termed the Warburg effect and has become a hallmark of cancer.

Despite the long history of research since Warburg's discovery, the pathways governing metabolic reprogramming and the connections between metabolism and immunity are now just beginning to be elucidated.

NF- κ B transcription factors

The nuclear factor- κ B (NF- κ B) family of dimeric transcription factors is comprised of five members: NF- κ B1 (p105/p50), NF- κ B2 (p100/p52), RelA/p65, RelB, and c-Rel (Hayden et al., 2008; Perkins, 2012). They are key regulators of innate and adaptive immunity but also play pivotal roles in essential cellular processes such as cell proliferation, differentiation, stress response, and survival (Grivennikov et al., 2010; Di Donato et al., 2012). NF- κ B proteins are present in all cell types and are normally held in an inactive state through binding to another family of related proteins named inhibitors of NF- κ B (I κ Bs). Similar to I κ Bs, p105 and p100 also possess a series of ankyrin repeats that allow them to serve as inhibitors of other NF- κ B member proteins. NF- κ B proteins are activated by a variety of signals, including cellular stress, DNA damage, immune receptor activation, bacterial and viral products, and inflammatory cytokines (Perkins, 2007). These signals lead to

activation of the I κ B kinases (IKKs), which in turn phosphorylate I κ B proteins bound to NF- κ B dimers, causing them to fall off and are degraded through the ubiquitin pathway, and result in the release of active NF- κ B dimers. Activated NF- κ B dimers then enter the nucleus and activate the expression of their target genes (Perkins, 2012). NF- κ B activity is tightly controlled through a number of negative feedback loops such as synthesis of I κ B proteins. This limits the time and extent of NF- κ B responses such as inflammation. Consequently, aberrant NF- κ B activation and inactivation can cause or contribute to many diseases (Karin, 2009; Bradford et al., 2014). A number of inflammatory diseases are driven by aberrant expression of inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin 6 (IL-6), which are both under the transcriptional control of NF- κ B. Due to the association between inflammation and cancer, NF- κ B can also contribute to the development of inflammation-associated cancers (Karin, 2009). In most cases, abnormal NF- κ B activity is driven either by pathological microenvironment, or by mutations or dysregulation of upstream regulators such as the Ras family of proto-oncogenes (Bradford et al., 2014). The target genes of NF- κ B transcription factors include many regulators of cancer-related cellular processes, such as proliferation, apoptosis, angiogenesis and metastasis. Therefore, NF- κ B activity can directly or indirectly contribute to many aspects of cancer.

Several recent studies have demonstrated NF- κ B's significant involvement in metabolic diseases such as type 2 diabetes, and atherosclerosis (Baker et al., 2011; Solinas et al., 2010). This involvement reflects the close integration of evolutionarily

conserved immune and metabolic pathways. From the species perspective, the abilities to endure nutrient stress and to fight against pathogens are both fundamental requirements for survival (Hotamisligil et al., 2008). In addition, mounting an effective immune response requires significant energy investment. Therefore, a close coordination between immune response and metabolic modulation is essential in both processes (Hotamisligil et al., 2008). This coordination is partly achieved through integration of nutrient- and pathogen-sensing machineries (Medzhitov, 2008; Barton, 2008). For example, the pattern-recognition receptors Toll-like receptors (TLRs) TLR2 and TLR4 can sense both lipopolysaccharides (LPS) in bacterial walls as well as fatty acids (Kawai et al., 2007). Conversely, metabolic hormones such as leptin and nutrient sensors such as peroxisome proliferator activated receptor (PPAR) also regulate immune functions (Lago et al., 2008; Hotamisligil, 2010). Thus, it is not surprising that pathogens can interfere with metabolic regulation and that nutrients can induce immune responses. The NF- κ B signaling pathway serves as a key connection between inflammation and metabolism (Baker et al., 2011). As an example, a number of metabolic stress sensors and cytokine receptors including tumor necrosis factor (TNF) receptors, interleukin-1 (IL-1) receptor, and TLRs converge on IKK β , leading to NF- κ B activation and expression of inflammatory genes, such as TNF α , IL-1 β , and IL-6 (Solinas et al., 2010). Recent evidence also indicates that high-calorie diet can induce ER stress, causing NF- κ B activation and inflammatory response (Cai et al., 2005; Zhang et al., 2008; Chiang et al., 2009).

NF- κ B has been known to integrate many signals that drive cell activation and proliferation during immune response and oncogenesis (Di Donato et al., 2012; Perkins, 2012; Kawauchi et al., 2008; Johnson et al., 2011). However, whether it directly regulates the metabolic reprogramming required to support cell division during these processes are just beginning to be answered. Recent reports indicate that there is a strong connection between NF- κ B and metabolism independent of inflammation (Mauro et al., 2011). The NF- κ B family member RelA has been shown to control energy metabolism through transcriptional regulation of metabolic genes, such as the glucose transporter GLUT3, thereby altering the balance between glycolysis and OXPHOS (Kawauchi, 2008). Because both immune response and tumor growth involve rapid cell division, it is not surprising that they both utilize increased glucose metabolism to meet the strong bioenergetic and biosynthetic demand. Situated at the focal point of signal integration, it makes sense for NF- κ B to coordinate the interrelated metabolic and immunological processes. Finally, although the NF- κ B family is known to play a crucial function in regulating a large number of genes involved in immune, developmental and apoptotic processes, the contributions of individual member proteins are still elusive.

NF- κ B family member c-Rel

The NF- κ B family member c-Rel (encoded by the REL gene in humans) was first identified as a homologue of the avian retroviral oncoprotein v-Rel, which causes

lymphoma in birds (Gilmore, 2011). Structurally, c-Rel is similar to RelA and RelB in the NF- κ B family, containing an N-terminal DNA-binding and dimerization domain, termed the Rel homology domain (RHD), and a C-terminal transcriptional activation domain (Gilmore, 2006; Martin et al., 2000 and 2001; Starczynowski et al., 2003). c-Rel is commonly found as a dimer with p50, RelA, or c-Rel itself, each carrying a slightly different DNA-binding specificity (Sanjabi et al., 2005). c-Rel is generally considered a transcriptional activator that promotes a permissive chromatin structure at the regulatory region of its target genes (van Essen et al., 2010).

Unlike other NF- κ B family members which are constitutively expressed in multiple cell types, c-Rel is expressed primarily in cells of lymphoid and myeloid origin, including B and T cells (Brownell et al., 1987; Huguet et al., 1998; Kontgen et al., 1995). In mouse, c-Rel has been shown to regulate the expression of many proinflammatory genes such as Il2, Tnf, Ifng, Il1b and Il6 (Pahl, 1999; Natoli et al., 2005; Carmody et al., 2007). c-Rel knockout mice show several immunological defects, including reduced mature B- and T-cell proliferation and activation in response to mitogenic stimuli and abnormal germinal center formation (Gilmore and Gerondakis, 2011). c-Rel also plays an important role in a number of autoimmune diseases such as inflammatory bowel disease and rheumatoid arthritis (Wang et al., 2008). c-Rel knockout mice are resistant to a number of autoimmune diseases, such as experimental autoimmune encephalomyelitis (EAE) and colitis (Campbell et al., 2000; Hilliard et al., 2002). c-Rel is also a key player in the development of suppressive regulatory T cells and inflammatory Th17 cells through transcriptional

regulation of Foxp3 and ROR γ T, respectively (Carmody et al., 2007; Ruan et al., 2009 and 2010). In addition, T cells isolated from c-Rel knockout mice fail to proliferate upon anti-CD3 or phorbol myristate acetate (PMA)/ionomycin stimulation (Liou et al., 1999). However, this phenotype was attributed to a deficiency in interleukin 2 (IL-2) production insofar as supplementation of exogenous IL-2 rescued the proliferation defect.

c-Rel in diseases

c-Rel has been linked to a number of human diseases through genomic and cytogenetic studies. Polymorphisms at the REL locus are associated with several autoimmune diseases, including rheumatoid arthritis (Gregersen et al., 2009; Eyre et al., 2010; Varadé et al., 2011), Crohn's disease (Cho et al., 2011), celiac disease (Trynka et al., 2009), psoriasis (Strange et al., 2010), and primary sclerosing cholangitis (Janse et al., 2011). REL gene amplifications and increased c-Rel expression have been found in a variety of B and T cell malignancies, such as Hodgkin's lymphoma, diffuse large B-cell lymphoma (DLBCL), and Adult T-cell leukemia/lymphoma (ATLL) (Fukuhara et al., 2006; Mader et al., 2007; Staudt et al., 2007; Courtois et al., 2006; Ramos et al., 2007). However, in several animal studies, c-Rel^{-/-} mice have shown greater susceptibility to cancer models, including colitis-associated colon adenocarcinoma and H. felis-induced model of gastric cancer (Burkitt et al., 2013 and 2015). Given c-Rel's importance in modulating immune

functions, these studies suggest that in addition to its inflammation-related tumor-promoting properties, c-Rel can also help to suppress tumorigenesis through its proper immune-regulatory role to support immune surveillance. Nevertheless, mechanistically how c-Rel contributes to tumorigenesis as a pathogenic factor remains unclear.

Despite the importance of c-Rel in T cell functions and its implications for diseases, the specific role it plays in metabolic regulation has not been identified. The elucidation of c-Rel's role in energy metabolism and its significance in cancer development will not only advance our knowledge of how NF- κ B functions in cancer, but also open new opportunities for identifying better targeting strategies of therapeutic intervention. Finally, because c-Rel is expressed preferentially in cells of lymphoid and myeloid lineages, drugs targeting c-Rel could have significantly less side effects than drugs targeting the other components or upstream regulators of the NF- κ B pathway.

CHAPTER 2: c-Rel Is a Key Regulator of T Cell Proliferation under Nutrient Stress

Introduction

The NF- κ B family member c-Rel was first discovered as a homologue of the avian retroviral oncoprotein v-Rel. It is also the only NF- κ B family member that can transform primary chicken spleen cells in vitro (Gilmore, 1999 and 2001). c-Rel is also unique in its preferential expression in lymphoid and myeloid cells, and its specific roles in B and T cell differentiation and function: c-Rel deficient mice exhibit reduced B cell activation, proliferation and survival, as well as defective Th1 response and Treg/Th17 development (Rao et al., 2003; Mason et al., 2004; Ruan et al., 2009 and 2010; Isomura et al., 2009).

Several pieces of evidence have implicated c-Rel in a number of human diseases. First, genome-wide association studies (GWAS) studies have linked polymorphism at the human REL locus to many autoimmune diseases (e.g., rheumatoid arthritis, psoriasis and celiac disease) and blood cancers (e.g., diffuse large B-cell lymphoma and Hodgkin's lymphoma) (Gilmore and Gerondakis, 2011). Second, REL gene amplifications and increased c-Rel expression have been found in a variety of B and T cell malignancies, such as Hodgkin's lymphoma, diffuse large B-cell lymphoma (DLBCL), and Adult T-cell leukemia/lymphoma (ATLL) (Hunter et al., 2016). And third, c-Rel knockout mice are resistant to models of RA, multiple

sclerosis (MS), type I diabetes and colitis (Gilmore and Gerondakis, 2011). Despite these implications, the questions of if and how c-Rel functions as a pathogenic factor remain unanswered. In addition, because most of the current knowledge on c-Rel is derived from mouse studies, whether they hold true in human remain to be seen.

Given the potential involvement of NF- κ B in T cell and cancer metabolism as described in Chapter 1, we decided to investigate the specific functions of c-Rel in human T cells with an emphasis on cancer- and metabolism-related processes.

In biology, the ideal first step towards understanding the function and importance of a protein is to remove its activity by knocking out its encoding gene and assess the impact. The results will lay the foundation for further investigations. Although c-Rel knockout mice have been generated for some time, to date there has been no report of human based c-Rel knockout cells being created or analyzed. With the recent advent of the CRISPR/Cas9 gene editing system, we decided to take advantage of this promising new technology to create the first human-based c-Rel knockout cell line. The first step of creating a knockout cell line is to select a suitable cell line as the model system and starting point. Based on our goal of studying c-Rel function in T cells, the ideal cell line should meet several criteria. First, it should be of T cell origin. Second, it should retain as much T-cell specific phenotype as possible. And third, it should have significant c-Rel expression. The human T cell leukemia line Jurkat meets all three criteria and was therefore selected as our model system.

Results

Generating c-Rel knockout (KO) Jurkat cells using the CRISPR/Cas9 system

To determine the specific functions of c-Rel in human T cells, we generated c-Rel knockout lines from the Jurkat human leukemia T cells using a CRISPR/Cas9 system. This specific system is comprised of two lentiviral-based vectors (Fig. 1A). The first vector (pCW-CAS9) carries the Cas9 endonuclease under the control of a Tet-On tetracycline inducible expression system, as well as a constitutively expressed puromycin resistance gene. The second vector (pLX-sgRNA) carries a single-guide RNA (sgRNA) driven by the U6 RNA polymerase III promoter, as well as a blasticidin resistance gene. The main advantage of this two-vector system over most one-vector based systems is that the Cas9 endonuclease is expressed only when doxycycline is added to the culture medium. The level of Cas9 will go down following the withdrawal of doxycycline from the culture media after gene editing has been completed. This helps to maintain genomic stability of the knockout cells by preventing unintended mutations created by excess amount of Cas9 protein. Our goal is to create frame-shift mutations near the N-terminus of the c-Rel coding sequence, thereby eliminating the translated c-Rel protein in its entirety. To achieve this goal, we designed eight sgRNA sequences, all targeting sites near the 5' end of the c-Rel coding sequence, and cloned them in to the pLX-sgRNA lentiviral vector. Five sgRNAs

whose target sites are closest to the 5' end of the c-Rel sequence were made into viruses and used to produce c-Rel KO cells (Fig 1B).

We first transduced the wild-type Jurkat (WT) cells with the pCW-Cas9 vector, followed by one week of puromycin selection and one week of expansion. The selected cells were then transduced again with the pLX-sgRNA vector followed by one week of blasticidin selection in the presence of doxycycline. The remaining cells were expanded to suitable numbers and analyzed for knockout efficiency using western blotting. The result indicated that two sgRNA sequences (#5 and 6) produced notable knockdown effect, while a third sequence (#2) showed weaker effect. One phenotype we noticed immediately was that cells transduced with sgRNA #5 and #6 grew much slower than cells transduced with the other sgRNAs. Because the sgRNA #6 group showed the strongest knockdown, we measured its growth rate against control groups (Fig. 2B). The growth curve indeed confirmed our initial observation.

The cells obtained by the end of blasticidin selection and doxycycline treatment are heterogeneous populations of c-Rel KO and non-KO cells. Because there appears to be a difference in growth rate between c-Rel KO and non-KO cells, the composition of these populations is unstable. We therefore proceeded with creating homogeneous c-Rel KO lines through clonal selection. The clonal selection gave rise to three c-Rel KO lines from different sgRNA groups and three sgRNA control clones (Fig. 3A), which were used in subsequent comparisons.

Because the critical dependence of IL-2 induction on c-Rel has been well established in mice, we measured IL-2 mRNA induction using quantitative PCR (qPCR) for functional confirmation of successful c-Rel knockout. The results confirmed that all three c-Rel KO lines failed to induce IL-2 expression upon PMA/ionomycin stimulation, while three control cell lines created through the same processes, but with an AAVS1-targeting control sgRNA, strongly expressed IL-2 upon stimulation (Fig. 3B).

The clonal selection process inevitably introduces selection bias in terms of growth rate. That is, non-proliferating or slow-proliferating cells will not come out of the selection. As a result, the growth rate differences observed before clonal selection growth were no longer evident when grown under standard culture medium containing 10% fetal bovine serum (FBS). However, upon placing the cells under nutrient stress by lowering serum concentration in the culture medium, all three c-Rel KO lines showed significantly lowered growth and survival rates (Fig. 4 and Fig. 5A). We therefore selected 2% FBS concentration as our standard serum starvation condition.

To confirm that this difference is due to c-Rel deletion and not to off-target effects of the CRISPR/Cas9 system, we re-expressed c-Rel in the KO cells under the control of the human Ubiquitin C (UbC) promoter using a lentiviral vector that also co-express GFP under the control of an internal ribosome entry site (IRES) element (Fig. 6A) for FACS-based selection (Fig. 6B and C). Western blotting confirmed

successful re-expression of the c-Rel protein, albeit at a higher level than the wt and sgRNA control groups. This ectopic expression of c-Rel rescued approximately 70% of the proliferation defect at day 6 (Fig. 7B), suggesting that the proliferation defect is indeed mostly attributable to c-Rel ablation.

c-Rel KO cells show impaired glucose and glutamine utilization

To find out which components in the medium were involved in the proliferation defect of the c-Rel KO cells, we removed individual components from the standard medium and measured the effects by measuring cell growth. Glucose and glutamine are two major energy sources used by cancer and normal proliferating cells. Using glucose-free base medium with or without the addition of glucose, we found that while the supplementation of glucose restored growth of the control cells under serum starvation, it had no effect on the c-Rel KO cells (Fig. 8A). Similarly, the addition of glutamine to glutamine-free (but not glucose-free) base medium markedly enhanced the growth of control cells, but again had no effect on the c-Rel KO cells (Fig. 8B). These results suggest that while normal Jurkat cells can utilize glucose and glutamine to support their growth and proliferation, the c-Rel KO cells have lost this ability regardless the availability of these nutrient sources.

c-Rel is required for c-Myc induction upon PMA/ionomycin stimulation or serum starvation

Given c-Rel's role as a transcription factor and the defects in glucose and glutamine utilization of c-Rel KO cells, we hypothesized that c-Rel facilitates cell proliferation by mediating the induction of genes crucial for metabolic reprogramming during nutrient stress. Because PMA/ionomycin stimulation is a well-established method to reliably elicit strong c-Rel activation, we decided to use this condition to screen for potential c-Rel targets that are also metabolic regulators involved in nutrient stress response. Using a high throughput microarray, we analyzed the transcriptome of normal and c-Rel KO Jurkat cells upon PMA/ionomycin stimulation. The results revealed downregulation of many metabolism-related genes in the c-Rel KO cells. We subsequently confirmed a number of these genes under serum starvation conditions via qPCR (Fig. 10). We also looked for potential c-Rel targets using previous c-Rel-deficient mouse B cell and macrophage microarray data sets, and cross-referenced the results to produce a Venn diagram (Fig. 11). We found that three genes: ACAT2, DHCR24, and c-Myc are common c-Rel targets in all three cell types of both human and mouse origin. Our qPCR confirmation experiments also showed that c-Myc mRNA expression is significantly induced in response to PMA/ionomycin stimulation, and that this induction is completely abolished in the absence of c-Rel (Fig. 12A). Furthermore, when c-Rel is re-expressed in the c-Rel KO cells, c-Myc induction is also restored (Fig. 12B). These results confirmed that c-Myc is indeed a c-Rel target in Jurkat T cells.

Next, we examined whether c-Myc is upregulated under serum starvation in a c-Rel-dependent manner. Using Western blots, we found that both c-Rel and c-Myc protein expressions are increased in a time-dependent manner under serum starvation (Fig. 13A). Importantly, this c-Myc induction is compromised at both the mRNA and the protein levels in c-Rel KO cells (Fig. 13B and Fig. 14A). Again, this defect can be rescued by re-expressing c-Rel in the c-Rel KO cells (Fig. 14B). Taken together, these results confirmed that c-Rel controls the induction of c-Myc expression during serum starvation.

The 2kb c-Myc promoter region is sufficient for response to c-Rel activation

The above results suggest that c-Rel might directly control c-Myc induction through transcriptional regulation at the c-Myc promoter. To evaluate this possibility, we cloned a 2kb genomic fragment containing the c-Myc promoter and inserted it upstream of a luciferase reporter gene (pGL4.12[luc2CP]). We then tested the responsiveness of this reporter construct to c-Rel activation using control and c-Rel KO Jurkat cells. The results showed that the c-Myc promoter conferred significant transcriptional activity upon PMA/ionomycin stimulation in the presence of c-Rel. However, this activity was significantly reduced by c-Rel knockout (Fig. 15). These data are consistent with our hypothesis that c-Rel activates c-Myc expression through transcriptional activation of the c-Myc promoter, which likely contains c-Rel-mediated DNA regulatory elements.

c-Myc reverses the cell growth defect of c-Rel KO cells

We went on to hypothesize that the c-Rel-dependent c-Myc induction is required to drive cell proliferation under nutrient stress. To test this hypothesis, we ectopically overexpressed c-Myc in c-Rel KO Jurkat cells under the control of the human Ubiquitin C (UbC) promoter using a lentiviral vector that also co-express GFP under the control of an internal ribosome entry site (IRES) element (Fig. 16A) for FACS-based selection (Fig. 16B and C). Western blotting showed that this ectopic expression increased c-Myc expression at both the mRNA and the protein levels (Fig. 17). More importantly, restoring c-Myc expression also reversed the proliferation defect of c-Rel KO cells under serum starvation (Fig. 18). These results suggested that the induction of c-Myc expression under serum starvation plays an important role in maintaining cell growth and proliferation.

Discussion

Our data not only confirmed that c-Rel is a critical factor for the proliferation and survival of Jurkat cells under nutrient stress, but also showed that a significant portion of this role involves transcriptional upregulation of the proto-oncogene protein c-Myc. c-Myc is a known contributor to the genesis of many human cancers (Pelengaris et al., 2002; Adhikary and Eilers, 2005) and has been shown to regulate

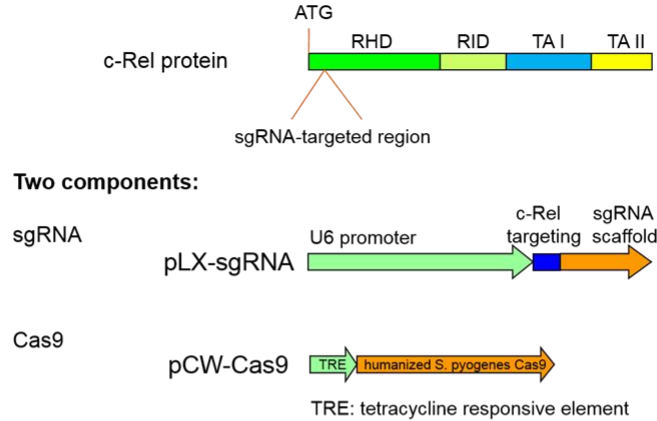
a large number of genes involved in cell growth, proliferation, differentiation, apoptosis as well as metabolism (Levens, 2002 and 2003). Indeed, our microarray and qPCR data confirmed a number of metabolism-related genes that are also thought to be c-Myc targets. Examples of these metabolic genes include the glucose transporter GLUT1, the mitochondrial transcription factors TFAM and PPARGC1A, as well as the lipid biosynthesis enzyme SLC27A2 (Fig. 10). Having shown that c-Rel activation can lead to c-Myc induction (Fig. 12), it would not be surprising that prolonged activation of c-Rel under inflammatory or other pathological conditions may cause persistent c-Myc upregulation, thus increasing the chance of tumorigenic transformation. Therefore, c-Myc may serve as a downstream effector of c-Rel that links altered cellular metabolism to tumorigenesis.

Mechanistically, c-Myc has been proposed to function as a universal amplifier of already expressed genes in lymphocytes and tumor cells (Nie et al., 2013; Lin et al., 2012). Chromatin immunoprecipitation coupled to high-throughput sequencing (ChIP-Seq) studies suggest that instead of serving as an on-off switch for a new set of genes, c-Myc accumulates in the promoter regions of active genes and enhances the level of transcription within the cell's existing gene expression program. This mode of function implies that a pre-established permissive chromatin environment is required at c-Myc target promoters. Interestingly, c-Rel has been shown to drive chromatin remodeling and establish a permissive chromatin environment at c-Rel-target promoters (Rao et al., 2003; van Essen et al, 2010). Having confirmed that c-

Myc itself is also a target of c-Rel, it is conceivable that their combined actions could form a synergistic mechanism to drive strong induction of their common targets.

Although the present study clearly establishes the growth-deficiency phenotype of c-Rel knockout Jurkat cells under nutrient stress, some important questions remain to be answered. For instance, we still do not know if c-Rel is directly involved in the initial steps of oncogenic transformation. Although we propose that c-Rel may contribute to this process through upregulation of c-Myc, this has yet to be proven experimentally. The fact that Jurkat is already a cancer cell line makes it unsuitable for answering this question. Therefore, a different approach needs to be taken to fully assess c-Rel's oncogenic potential. Another interesting observation is that although re-expression of c-Rel in the knockout cells achieved much higher protein level as indicated by western blotting, it did not fully restore the growth defect of the c-Rel (Fig. 7). This shows that the level of c-Rel expression and the rate of cell growth are not in a simple linear relationship. The incomplete rescue of cell growth rate could be a side-effect of excessive c-Rel protein expression. Or, it could be that proper post-transcriptional regulation, such as alternative splicing into multiple isoforms, is required for c-Rel's normal function. These possibilities will need to be explored to fully explain this phenomenon.

A.



B.

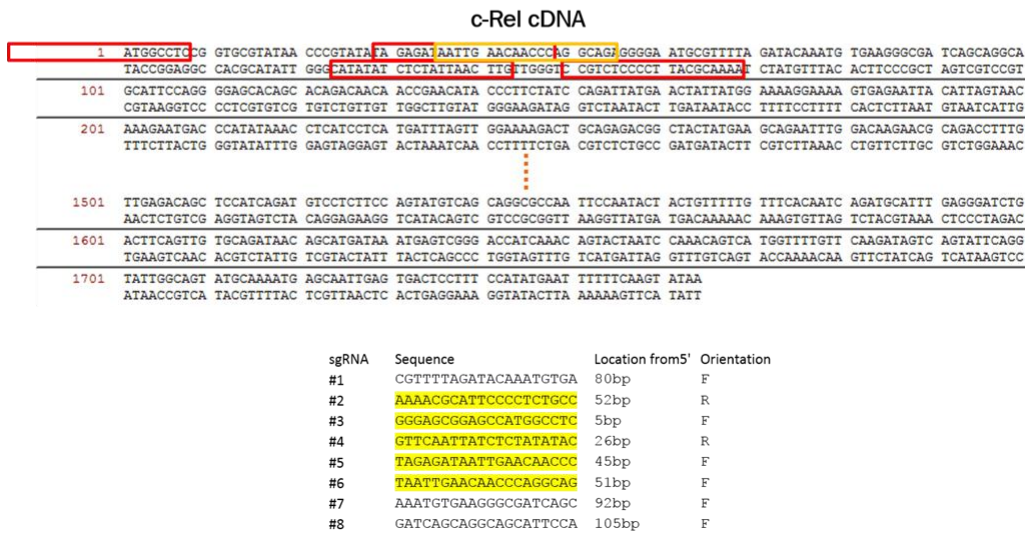
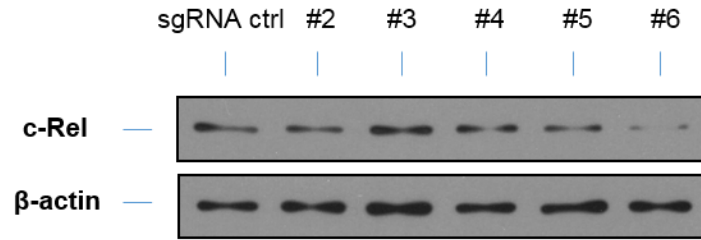


FIGURE 1. Design of a CRISPR/Cas9 system to create human c-Rel knockout cells

A two-component lentiviral based CRISPR/Cas9 system is used to sequentially deliver Cas9 endonuclease and single-guide RNA (sgRNA) into cells. A) sgRNAs are constitutively expressed under the control of the U6 promoter, while Cas9 is expressed using a tet-on system in a doxycycline-dependent manner. B) Five sgRNA sequences (highlighted) targeting N-terminus of the c-Rel protein were selected to generate c-Rel knockout cells. The exact locations of sgRNA targets are indicated on the c-Rel cDNA sequence map.

A.



B.

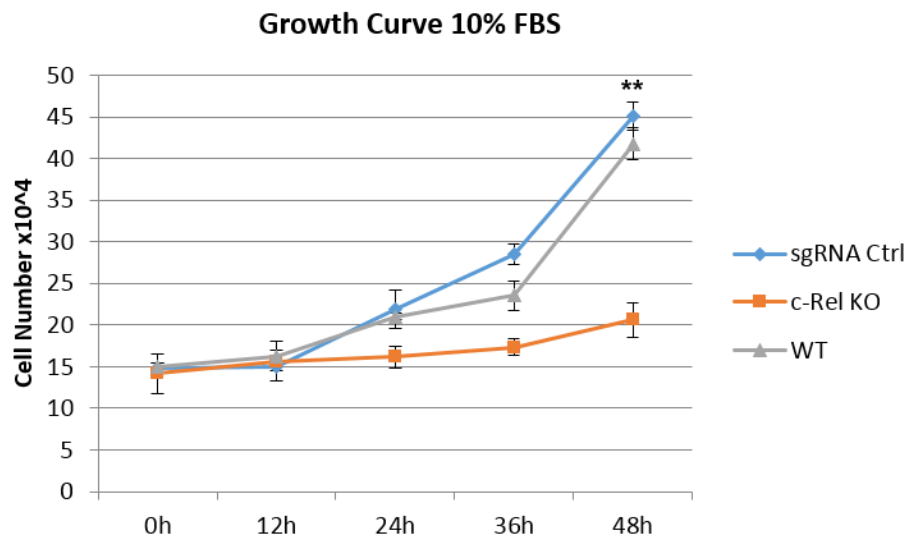
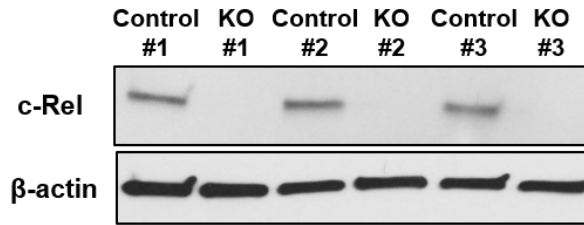


FIGURE 2. c-Rel knockout Jurkat cells show reduced cell growth

A) Western blots of Jurkat cells treated with Cas9 and five different c-Rel-targeting sgRNAs and an AAVS1-targeting control sgRNA. Notable knockdown effect of c-Rel was observed on cells treated with sgRNA #5 and #6. B) Cell growth of Jurkat cells treated with sgRNA #6 was measured against control sgRNA-treated and untreated (WT) Jurkat cells. Values represent mean \pm SEM (n=3; **p<0.01; one-way ANOVA).

A.



B.

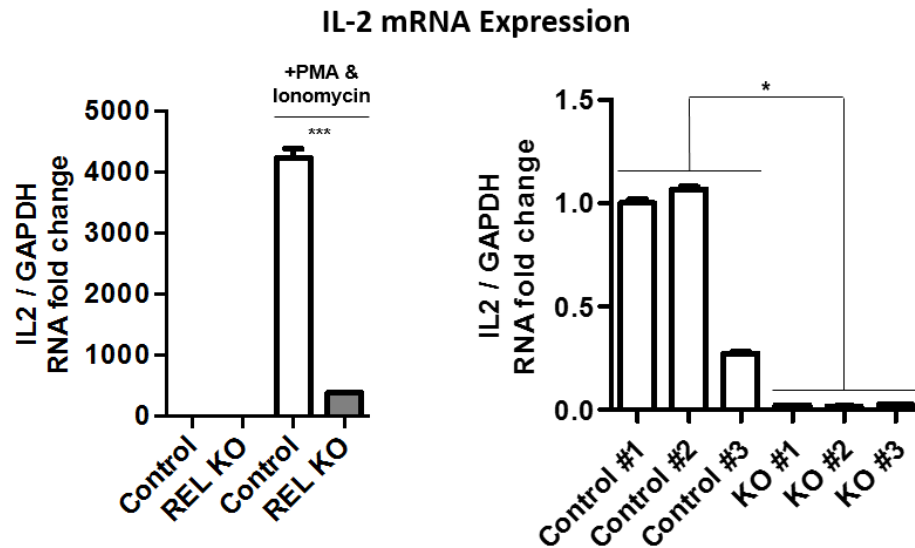
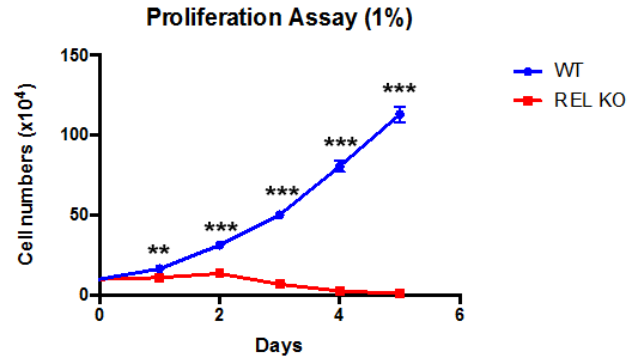


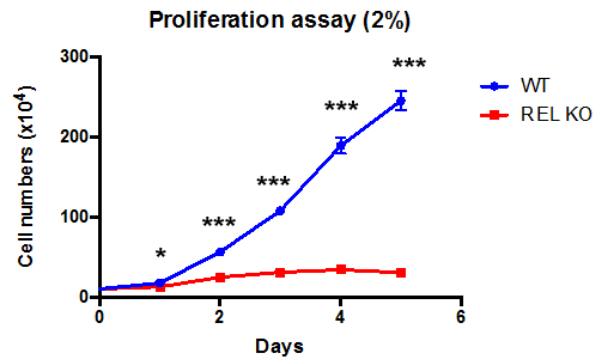
FIGURE 3. c-Rel knockout Jurkat cells fail to express IL-2 upon PMA / ionomycin stimulation

A) Western blots of three c-Rel knockout (KO) Jurkat clones and three sgRNA control clones. Complete knockout is observed in all three c-Rel KO clones. B) Control and c-Rel KO Jurkat cells were stimulated with or without PMA and ionomycin for 4 hours, followed by RT-qPCR assay for human IL-2. Left: Values represent mean \pm SEM of pooled samples of equal amounts of RNA from 3 clones ($n=3$; *** $p<0.001$; one-way ANOVA). Right: Values represent mean \pm SEM of individual clones ($n=3$; * $p<0.05$; Student's t-test).

A.



B.



C.

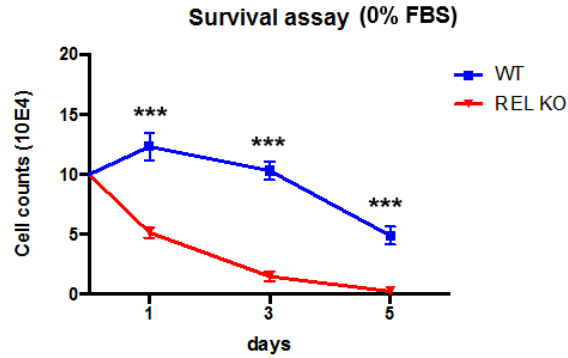
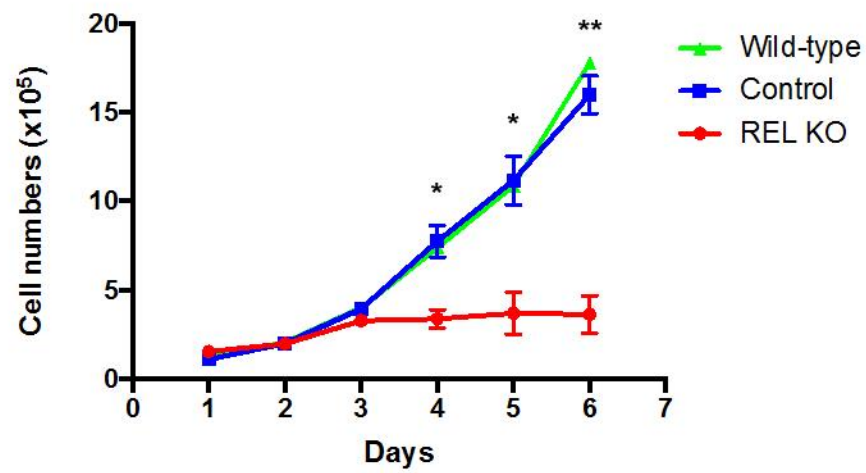


FIGURE 4. c-Rel KO Jurkat cells show severe growth and survival defect under low serum concentrations

A) wt and c-Rel KO Jurkat cells were cultured in medium containing 1% FBS for 5 days. B) wt and c-Rel KO Jurkat cells were cultured in medium containing 2% FBS for 5 days. C) wt and c-Rel KO Jurkat cells were cultured in serum-free medium for 5 days. Values represent mean \pm SEM of 6 technical replicates of one clone in each group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Student's t-test).

A.



B.

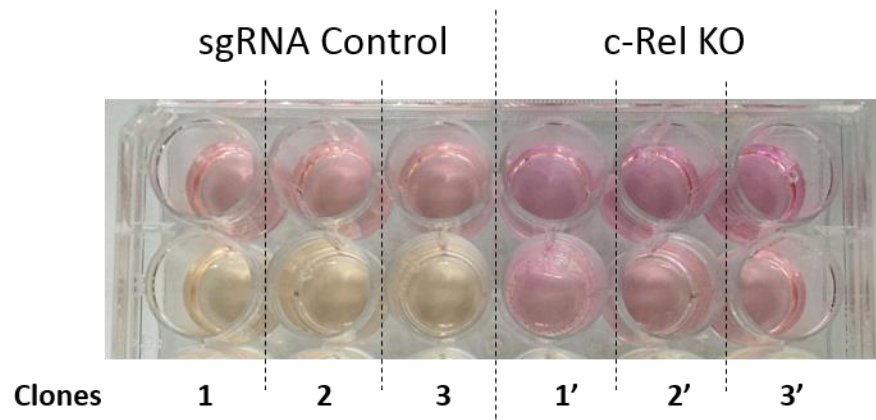


FIGURE 5. c-Rel is required for cell growth under serum starvation

A) sgRNA control (blue), c-Rel KO (red), and wild-type (green) Jurkat T cells were grown in culture media containing 2% FBS. Values represent mean \pm SEM of 3 independent clones in each group (*p<0.05, **p<0.01; Student's t-test). B) c-Rel KO cell groups show increased culture media acidity as indicated by phenol red color change after 5 days.

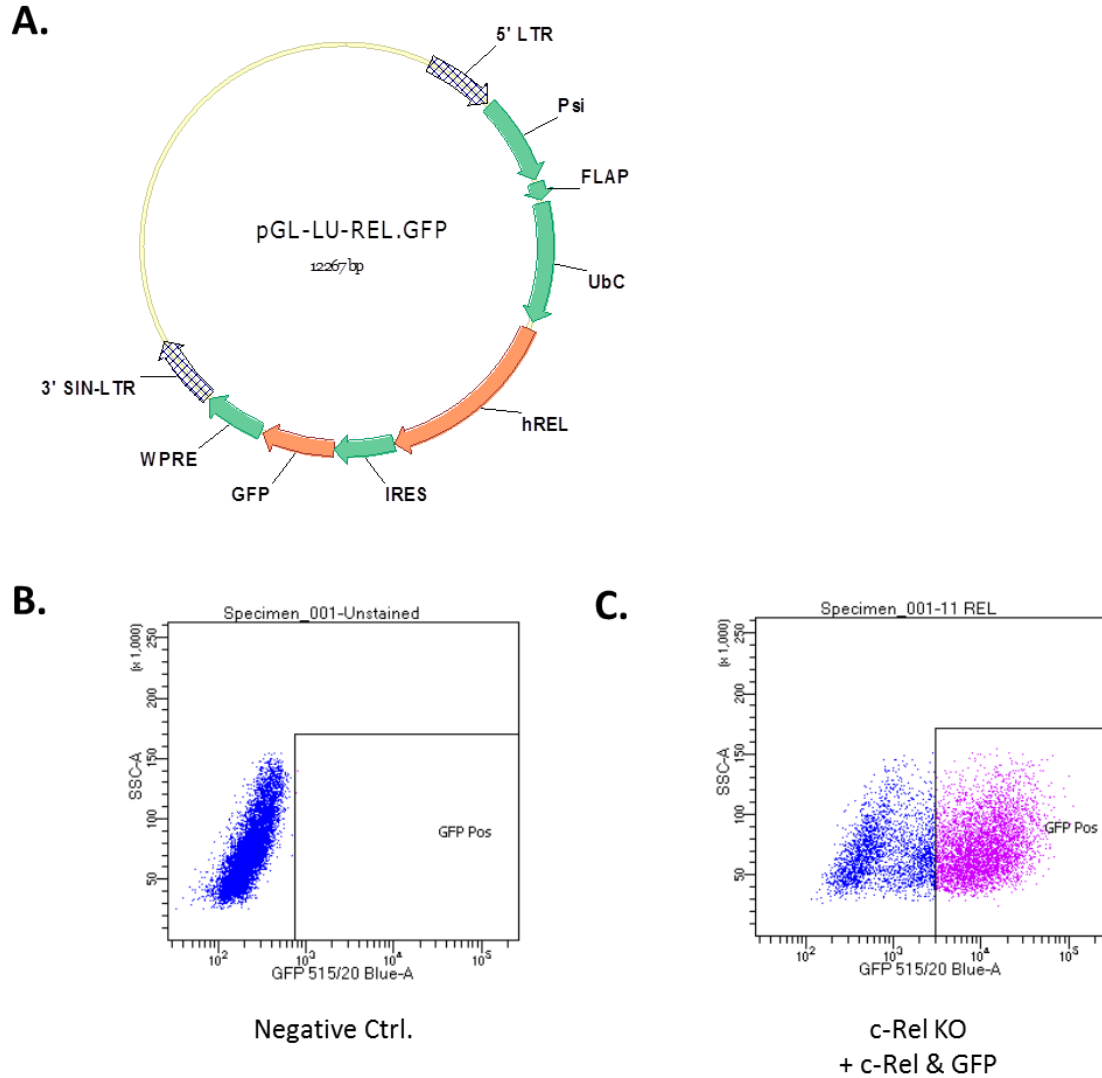


FIGURE 6. Rescuing c-Rel expression in c-Rel KO cells using a lentiviral vector

A) Schematic representation of the c-Rel expression lentiviral vector: Human c-el is expressed under the control of the ubiquitin C promoter. GFP is co-expressed after an internal ribosome entry site (IRES) element following the c-Rel cDNA sequence. B) Flow cytometry graph of c-Rel KO cells transduced with control vector. C) FACS sorting of c-Rel KO cells transduced with c-Rel and GFP as a marker.

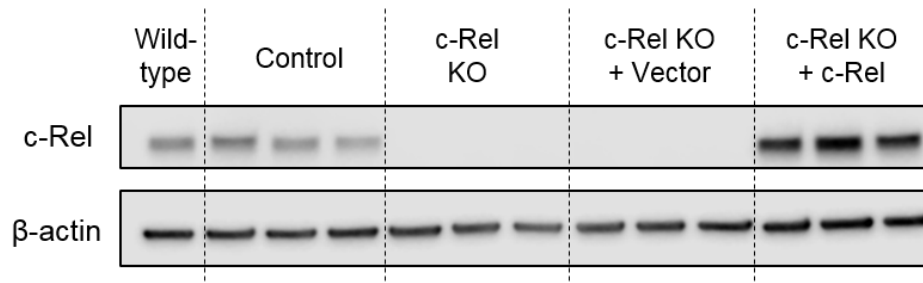
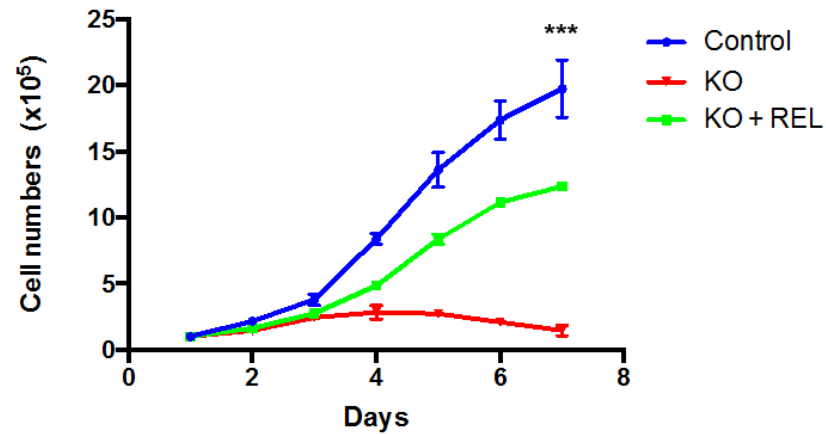
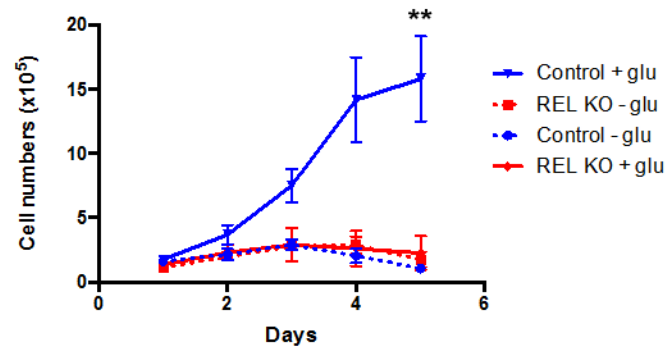
A.**B.**

FIGURE 7. Re-expressing c-Rel in c-Rel KO cells rescues their growth defect

A) Western blots from Jurkat cell clones of wild type (WT), sgRNA control (Ctrl), c-Rel KO, c-Rel KO transduced with GFP-expressing lentiviral vector, and c-Rel KO transduced with c-Rel and GFP co-expressing vector. Each blot represents a single clone. B) Growth curve of sgRNA control cells transduced with GFP (blue), c-Rel KO cells transduced with GFP (red), and c-Rel KO cells transduced with c-Rel and GFP (green) under 2% serum concentration. Values represent mean \pm SEM of 3 independent clones in each group (**p<0.01; one-way ANOVA).

A.



B.

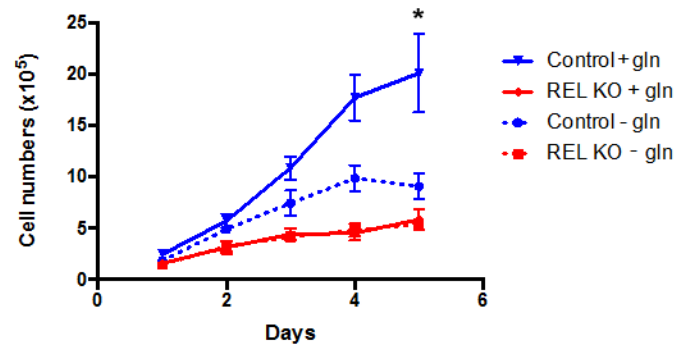


FIGURE 8. c-Rel is required for glucose and glutamine utilization

A) Growth curve of sgRNA control and c-Rel KO Jurkat cells in culture media with or without glucose (glu, 11mM) under 2% serum starvation conditions. B. Growth curve of sgRNA control and c-Rel KO cells in culture media with or without glutamine (gln, 2mM) under 2% serum starvation conditions. For all panels, values represent mean \pm SEM of 3 independent clones in each group (p values for control cells without glu/gln vs control cells with glu/gln: *p<0.05, **p<0.01; one-way ANOVA).

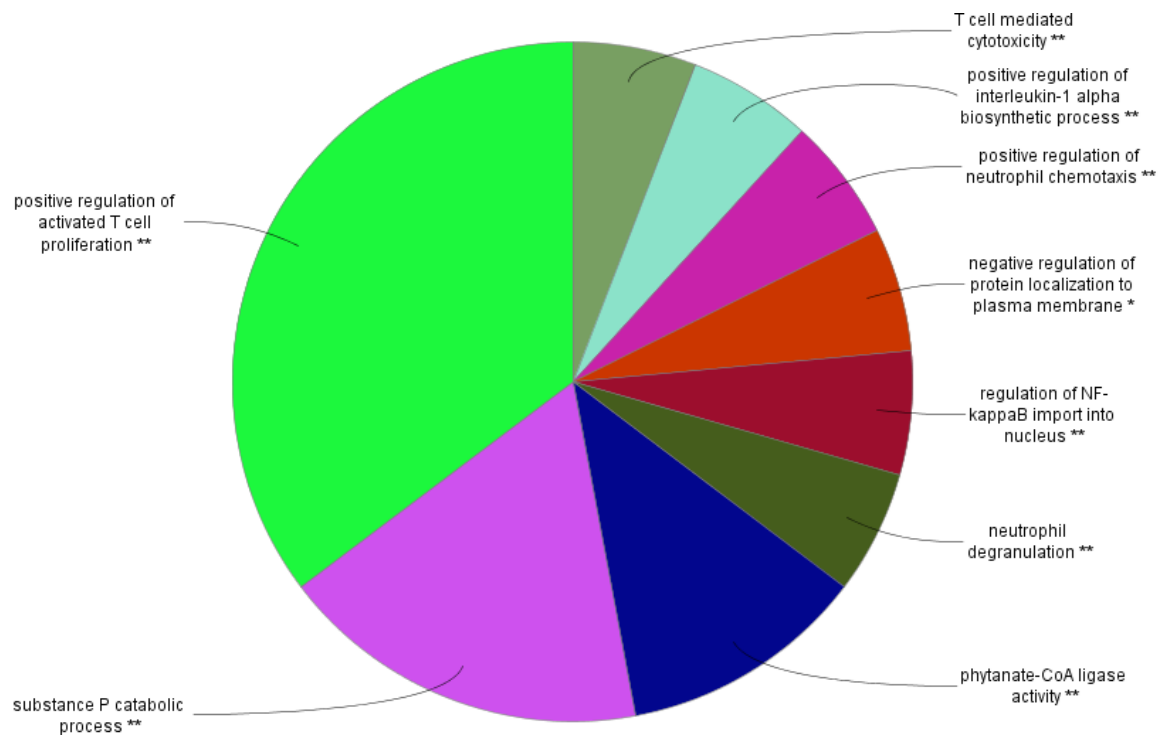


FIGURE 9. Microarray analysis showing significant biological processes affected by c-Rel knockout

Areas represent number of genes involved in the respective processes and are affected by c-Rel knockout, as identified through gene ontology functional annotation clustering analysis based on microarray results.

mRNA Level by qPCR

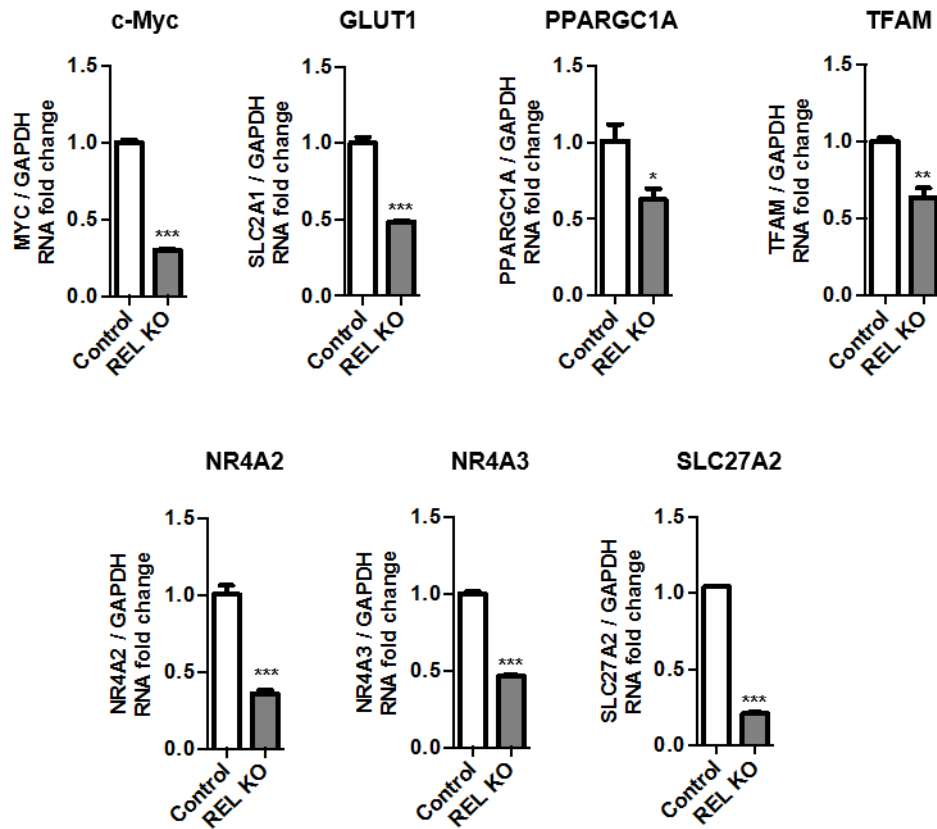


FIGURE 10. c-Rel knockout leads to downregulation of a number of metabolism related genes

Control and c-Rel KO Jurkat cells were cultured in standard culture media and stimulated with PMA and ionomycin for 4 hours, followed by RT-qPCR analyses of respective genes. For all panels, values represent mean \pm SEM of pooled samples of equal amounts of RNA from 3 clones (n=3; *p<0.05, **p<0.01, ***p<0.001; Student's t-test).

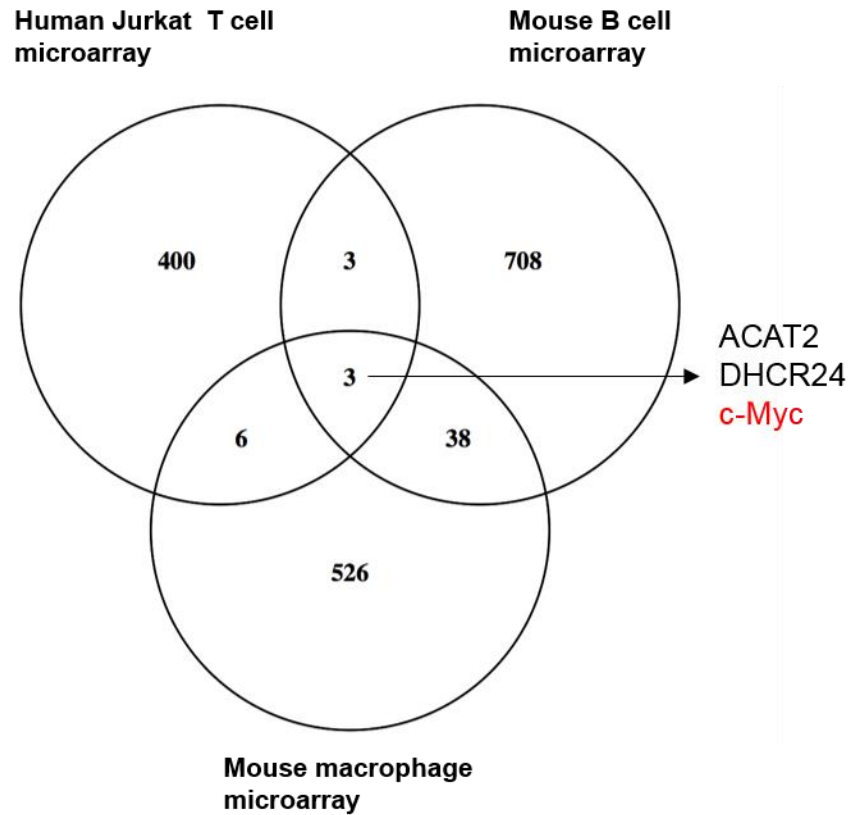
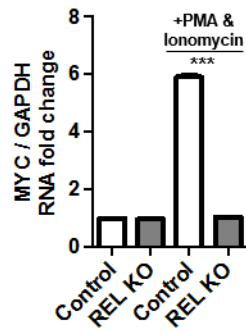


FIGURE 11. c-Myc is identified as a c-Rel target through microarray analyses.

Venn Diagram of 3 microarrays comparing c-Rel KO with control in human Jurkat T cells stimulated with TCR agonist PMA and ionomycin, mouse macrophages stimulated with lipopolysaccharide (LPS), and mouse B cells stimulated with BCR agonist CD40 and IgM.

A.



B.

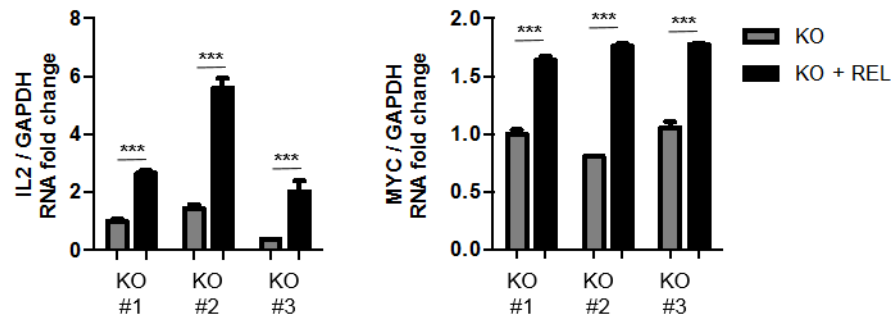
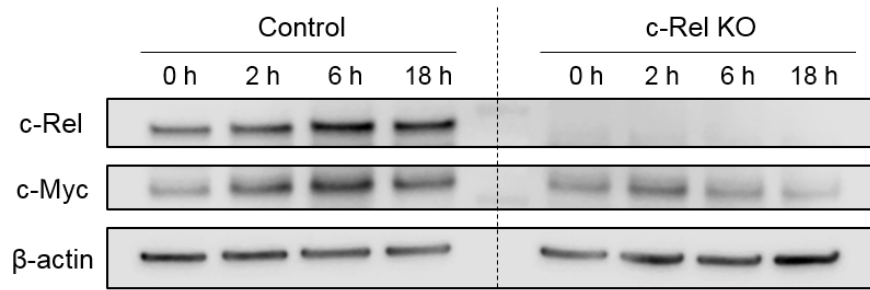


FIGURE 12. c-Rel is required for c-Myc induction under PMA/ionomycin stimulation

A) sgRNA control and c-Rel KO Jurkat cells were cultured in standard culture media and treated with or without PMA/ionomycin stimulation followed by RT-qPCR quantification of c-Myc mRNA. Values represent mean \pm SEM of pooled samples of equal amounts of RNA from 3 clones ($n=3$; *** $p<0.001$; one-way ANOVA). B) c-Rel KO cells transduced with GFP and c-Rel KO cells transduced with c-Rel and GFP were cultured in standard culture media and stimulated with PMA/ionomycin for 4 hours, followed by RT-qPCR quantification of IL-2 (left) and c-Myc mRNA (right). Values represent mean \pm SEM of individual clones ($n=3$; *** $p<0.001$; Student's t-test).

A.



B.

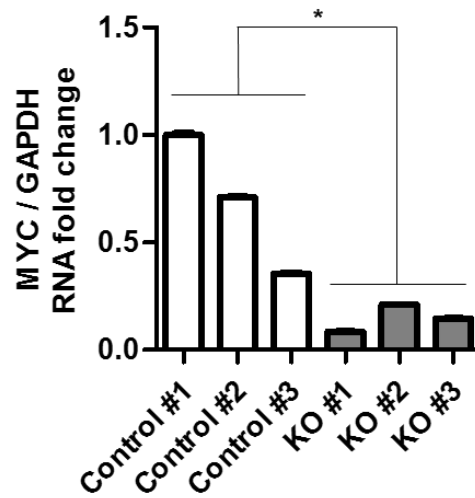
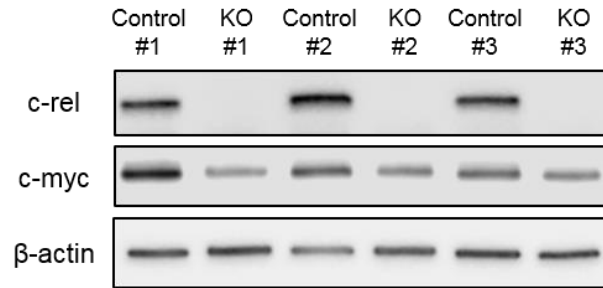


FIGURE 13. c-Rel knockout leads to deficiency in c-Myc induction under serum starvation

A) sgRNA control and c-Rel KO Jurkat cells were starved under 2% FBS for the indicated times followed by Western blotting for c-Rel and c-Myc. B) sgRNA control and c-Rel KO Jurkat cells were serum starved for 2 days followed by RT-qPCR quantification of c-Myc mRNA. Data were normalized with GAPDH. Values represent mean \pm SEM of individual clones (n=3; *p<0.05; Student's t-test).

A.



B.

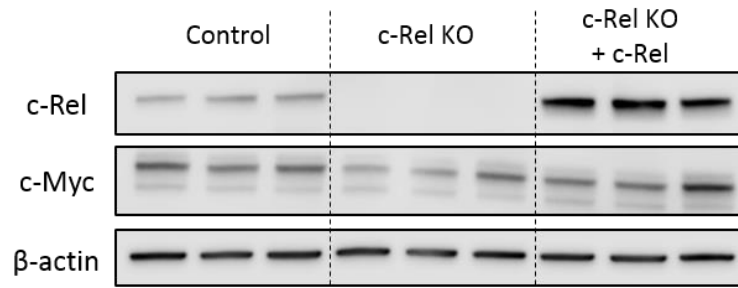


FIGURE 14. Re-expressing c-Rel in c-Rel KO cells restores c-Myc induction under serum starvation

A) wild-type (WT), sgRNA control (Ctrl), and c-Rel KO Jurkat cell clones were serum starved for 2 days followed by Western blotting. B) Clones of sgRNA control cells transduced with GFP, c-Rel KO cells transduced with GFP, and c-Rel KO cells transduced with c-Rel and GFP were serum starved for 2 days followed by Western blotting for c-Rel and c-Myc.

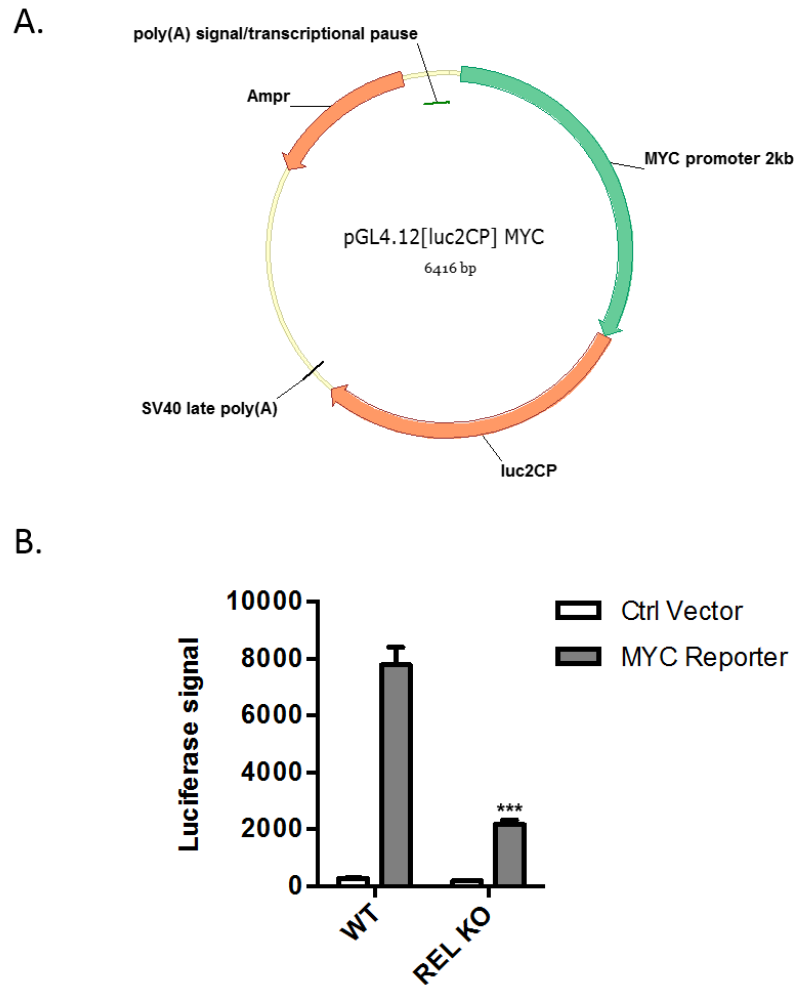


FIGURE 15. c-Rel drives c-Myc promoter activity

A) Schematic representation of the c-Myc promoter reporter plasmid: a 2kb human c-Myc is inserted upstream of the luciferase reporter gene luc2CP. B) Control and c-Rel KO Jurkat cells were transfected with a luciferase reporter plasmid containing either no promoter or a 2kb human c-Myc promoter, and stimulated overnight with PMA and ionomycin followed by luciferase assay. Values represent mean \pm SEM (n=3; p values for c-Myc reporter signal in WT vs in REL KO: ***p<0.001; one-way ANOVA).

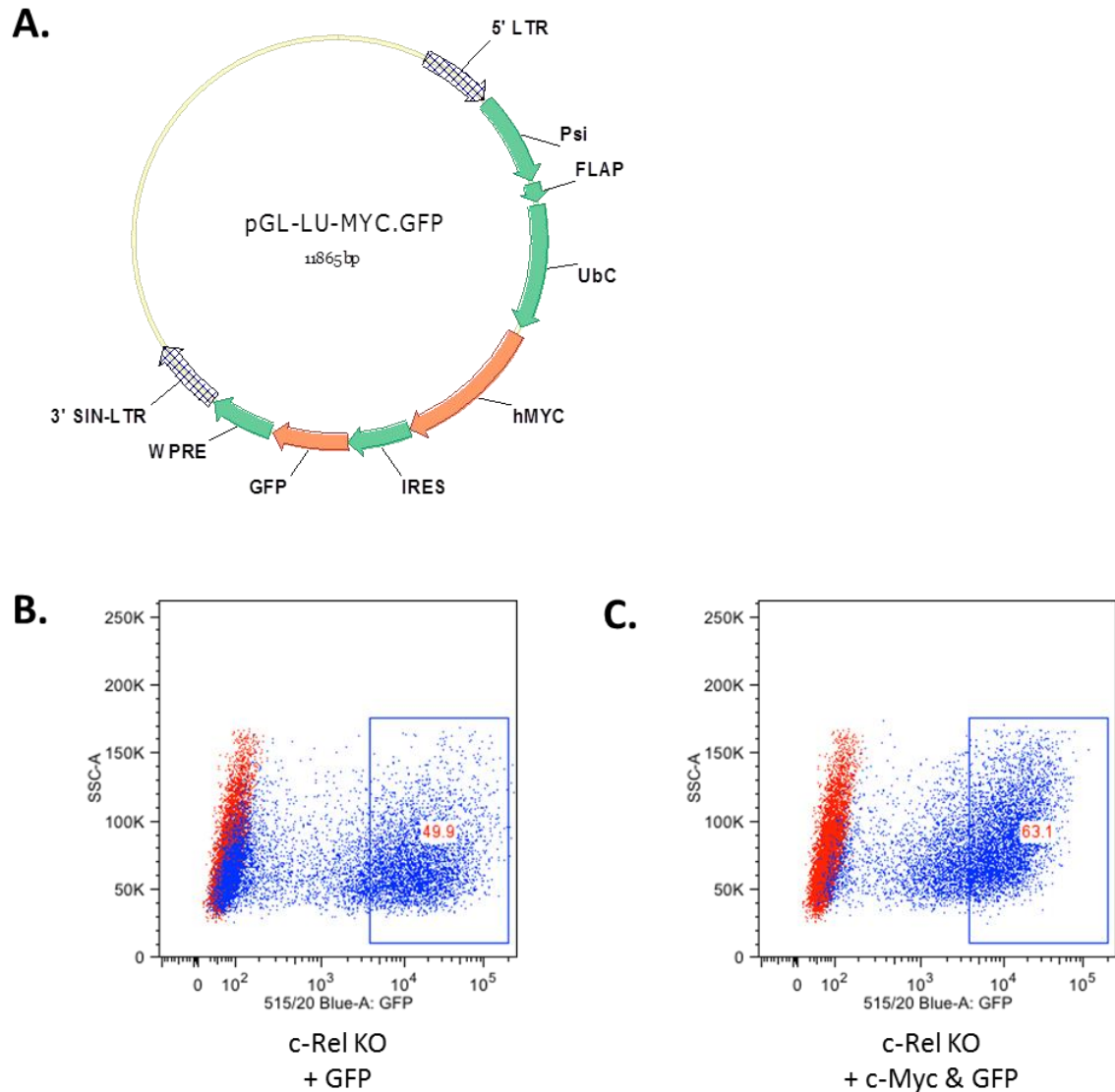


FIGURE 16. Rescue of c-Myc deficiency through expression of c-Myc

A) Schematic representation of the c-Myc expression lentiviral vector: Human c-el is expressed under the control of the ubiquitin C promoter. GFP is co-expressed after an internal ribosome entry site (IRES) element following the c-Rel cDNA sequence. B) FACS sorting of c-Rel KO cells transduced with GFP only. C) FACS sorting of c-Rel KO cells transduced with both c-Myc and GFP.

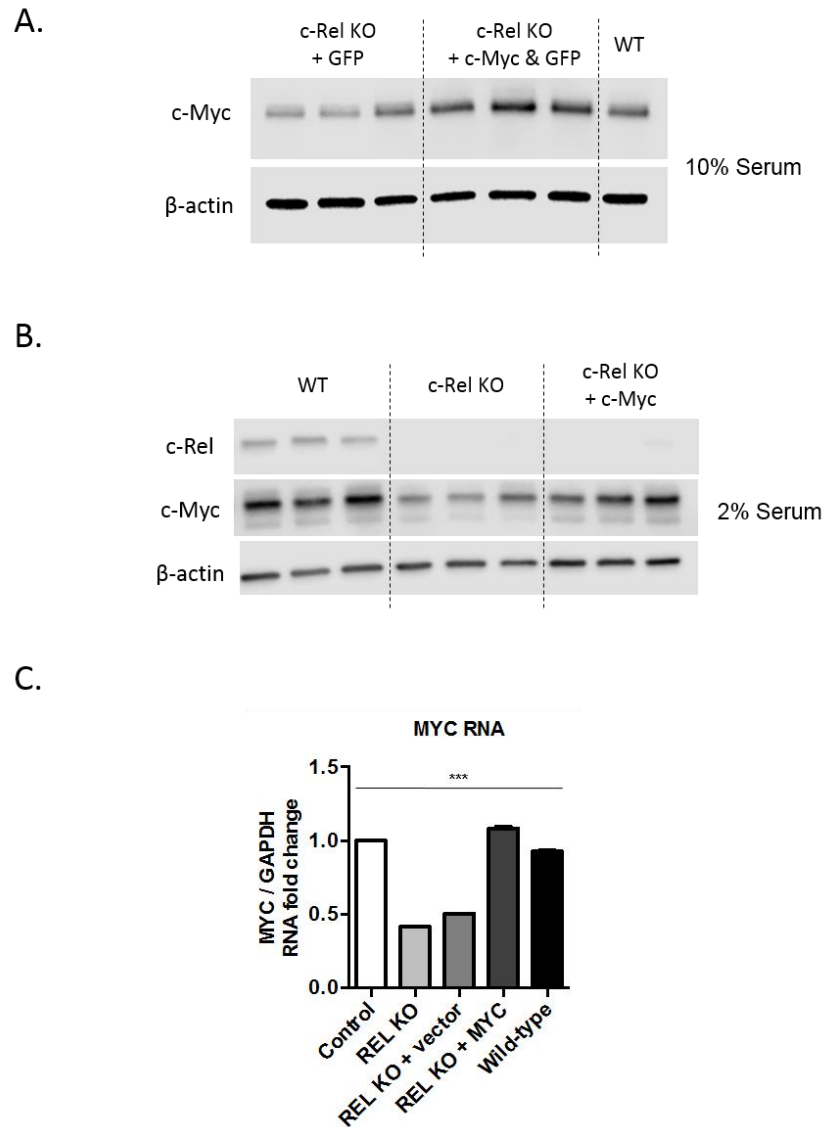


FIGURE 17. c-Myc expression compensates c-Myc induction failure in c-Rel KO cells

A) Western blots of c-Rel KO cells transduced with GFP, c-Rel KO cells transduced with c-Myc and GFP, and wild-type Jurkat cells that were cultured in standard media containing 10% FBS. Three independent clones in each group are shown. B) sgRNA control cells transduced with GFP, c-Rel KO cells transduced with GFP, and c-Rel KO cells transduced with c-Myc and GFP were starved under 2% FBS for 2 days followed by Western blotting. Three independent clones in each group are shown. C) The indicated cells were starved under 2% FBS for 2 days followed by RT-qPCR quantification of c-Myc. Data were normalized by GAPDH. Values represent mean \pm SEM of pooled samples of equal amounts of RNA from 3 clones (n=3; ***p<0.001; one-way ANOVA).

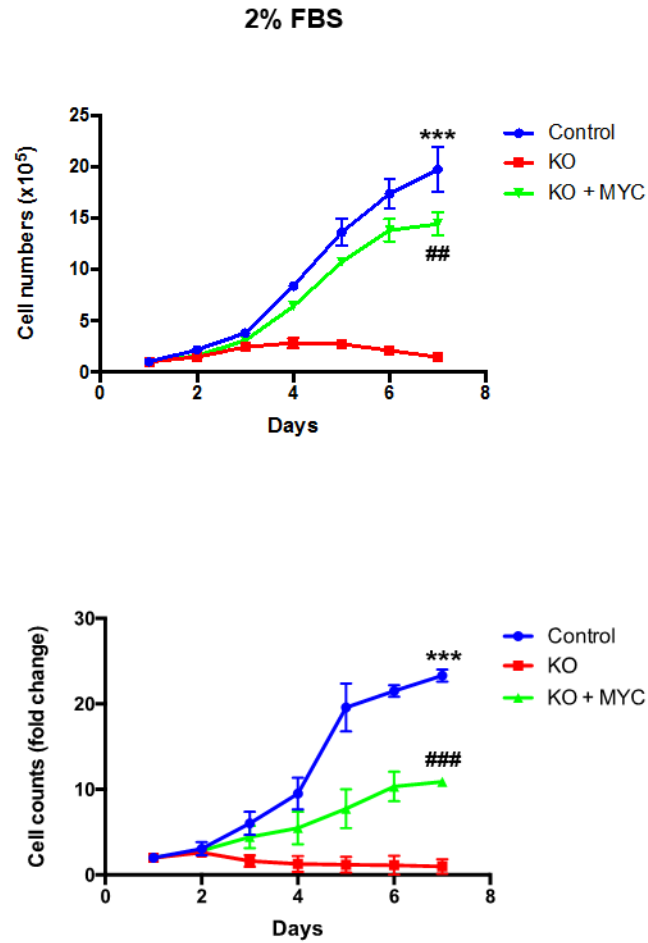


FIGURE 18. c-Myc rescues growth defect of c-Rel KO cells under serum starvation

Two independent measurements of growth curve of sgRNA control, c-Rel KO, and c-Rel KO cells transduced with c-Myc under 2% serum starvation. Values represent mean \pm SEM of 3 independent clones in each group (***Control vs KO, $p < 0.001$; ##KO+MYC vs KO, $p < 0.01$; ###KO+MYC vs KO, $p < 0.001$. one-way ANOVA).

CHAPTER 3: c-Rel Regulates T Cell Metabolism by Promoting Glycolysis and Mitochondrial Respiration

Introduction

Having confirmed that c-Rel is essential for maintaining cell under serum starvation, and that it controls c-Myc induction under the same condition, we want to further determine how much does the latter contribute to the former. Given that c-Myc is a known metabolic regulator (Wise et al., 2008; Gaber et al., 2015; Miller et al., 2012), it is natural to ask whether c-Rel regulates cell growth through metabolic modulation, and if so, does it modulate energy metabolism entirely through driving c-Myc induction? Another question is that because Jurkat is a transformed cancer cell line, can the knowledge we learn from Jurkat cells be applied to normal primary T cells? To answer these questions, we decided to perform detailed metabolic profiling of the c-Rel KO and the control cells. Since glycolysis and mitochondrial respiration are the two major sources of energy production used by cancer and normal cells, we put our emphasis on these two processes. We started by measuring glucose consumption in the culture as a pilot experiment, followed by detailed quantification of metabolic parameters of glycolysis and mitochondrial respiration using a Seahorse XF extracellular flux analyzer. Furthermore, we also isolated primary mouse CD4⁺ T cells from c-Rel KO and wild type mice, and measured their metabolic parameters using the Seahorse assay.

Results

c-Rel KO cells show reduced glucose consumption

We started by measuring the glucose consumption of sgRNA control cells, c-Rel KO cells, and c-Rel KO cells transduced with c-Myc expression vector. The result showed that the c-Rel KO cells consume less glucose than the sgRNA control cells, and that ectopic c-Myc overexpression can partially reverse this effect (Fig. 19). It is worth noting that this difference is not merely the results of different cell numbers, since this difference is already evident on day two, when cell numbers in the different groups were still roughly equal.

Glycolysis and mitochondrial respiration are severely compromised in c-Rel knockout cells

Activated T cells and cancer cells both rely on metabolic reprogramming that boosts processes such as glycolysis and glutaminolysis to support their growth and proliferation. Thus, it is important to know which functional aspects of these processes are controlled by c-Rel. We therefore performed detailed metabolic profiling to assess the metabolic phenotypes of the c-Rel KO Jurkat cells. To measure the metabolic parameters of the control and c-Rel KO Jurkat cells, we quantified the oxygen consumption and lactate production of these cells using an

extracellular flux analyzer (Seahorse XF). First, glycolysis was measured by lactate production as the extracellular acidification rate (ECAR). Upon addition of glucose, c-Rel KO cells showed significantly less increase in glycolysis than the control cells. Next, oligomycin was added to inhibit mitochondrial ATP synthesis and achieve maximum glycolytic capacity. Again, while the control cells showed a strong increase in ECAR, the c-Rel KO cells exhibited little change (Fig. 20). These results suggest that c-Rel KO cells have a deficiency in the glycolysis pathway, resulting in a significantly reduced flux and limited reserve capacity that could not meet the metabolic demand for proliferation under nutrient stress.

In addition to glycolysis, activated T cells and cancer cells also use TCA cycle and other metabolic pathways to enhance biosynthetic processes underpinning their growth and proliferation. We therefore measured mitochondrial respiration of the c-Rel KO cells by the oxygen consumption rate (OCR). The results revealed that before the electron transport chain inhibitors were added, the c-Rel KO cells showed significantly lower basal rate of oxygen consumption relative to the control cells. The addition of oligomycin suppressed oxygen consumption of both the c-Rel KO cells and the control cells to comparable levels, suggesting a tight coupling of oxygen consumption and ATP production in both groups. After FCCP was added to uncouple electron transport from oxidative phosphorylation, thus allowing for maximal oxygen consumption, the control cells increased their oxygen consumption to a level significantly above the basal rate. At the same time, c-Rel KO cells merely increased their oxygen consumption to the basal level (Fig. 21). These data revealed

that c-Rel KO cells not only have reduced basal rates of mitochondrial respiration but also have significantly reduced spare respiratory capacity (SRC).

c-Myc is partially responsible for the metabolic defects of c-Rel KO cells

Having shown that c-Rel-dependent c-Myc induction is important for the maintenance of cell growth under nutrient stress, and that c-Rel plays a key role in promoting glycolysis and mitochondrial respiration, we want to find out how much c-Myc accounts for the metabolic function of c-Rel. Indeed, results of the Seahorse assay confirmed that overexpression of c-Myc in c-Rel KO cells can rescue most of the mitochondrial respiration deficiency (Fig. 21). However, it only partially rescued the glycolytic defect (Fig. 20). Interestingly, qPCR quantification of the key glucose transporter GLUT1 revealed that c-Myc overexpression in c-Rel KO cells also only partially reversed the downregulation of GLUT1 in c-Rel KO cells (Fig. 22), and that the extent of rescue in GLUT1 expression roughly correlates with the extent of rescue in glycolysis.

Collectively, these results show that c-Myc makes up a significant, but not all part of the c-Rel-controlled metabolic modulation

c-Rel promotes glycolysis and mitochondrial respiration in primary T cells in mice

Finally, to find out whether our findings are broadly applicable to normal primary T cells, or only specific to the Jurkat cell line, we performed the same Seahorse metabolic profiling on primary CD4⁺ T cells isolated from WT and c-Rel KO mice (Fig. 23). The results again showed that both glycolysis and mitochondrial respiration are severely compromised in mouse c-Rel knockout T cells.

Discussion

Although Warburg's findings established that most cancer cells rely primarily on aerobic glycolysis to generate the energy needed for their cellular activities, it was later recognized that they also utilize OXPHOS (Weinberg et al., 2010; Fogal et al., 2010). Warburg had proposed that the aerobic glycolysis in cancer cells was caused by damage to the mitochondrial OXPHOS machinery. However, later studies have found that the function of the OXPHOS machinery is largely intact in most spontaneous tumors (Lim et al., 2011; Scott et al., 2011), and that the switch from OXPHOS to glycolysis is often a result of active metabolic reprogramming induced by oncogenic mutations or environmental stress (Koppenol et al., 2011). Relevant to our study, it has been shown that c-Myc plays an important role in mitochondrial biogenesis. A number of nuclear-encoded regulators of mitochondrial biogenesis and functions are targets of c-Myc (Menssen et al., 2002; Fernandez et al., 2003). Our qPCR results also confirmed downregulation of two key transcription factors for mitochondrial biogenesis – TFAM and PPARGC1A – in c-Rel KO cells. In fact, a

study using inducible c-Myc expression in human B cells showed that mitochondrial biogenesis is critically dependent on c-Myc expression (Li et al., 2005). Finally, the c-Myc driven mitochondrial synthesis of acetyl-CoA may serve to provide a strong support for fatty acid biosynthesis in proliferating cells.

In addition to c-Myc, the NF- κ B family member RelA has also been shown to regulate cellular metabolism. One study showed that in mouse embryo fibroblasts (MEFs), RelA can promote oxidative phosphorylation under glucose starvation by upregulating p53 expression, which in turn upregulates SCO2 – a crucial component of the mitochondrial cytochrome C oxidase (COX) complex that sits at the end of the electron transport chain (Mauro et al., 2011). Taken together, it may not be a surprise that c-Rel knockout leads to defects in both glycolysis and mitochondria respiration.

Having observed the impaired proliferation and reduced glucose consumption of the c-Rel KO cells, it is natural to question the causal relationship between the two phenotypes. However, there may not be a simple answer to this question, as each phenotype is influenced by multiple overlapping factors and is subject to complex feedback regulation. Nevertheless, the results from our metabolic flux profiling assays may provide some important insights. In particular, the data not only showed reduced glucose and oxygen consumption under standard conditions, but also revealed severe reductions in both total glycolytic capacity and spare respiratory capacity. These results suggest that in Jurkat T cells, c-Rel is itself

an integral part of the metabolic machinery rather than an indirect upstream regulator.

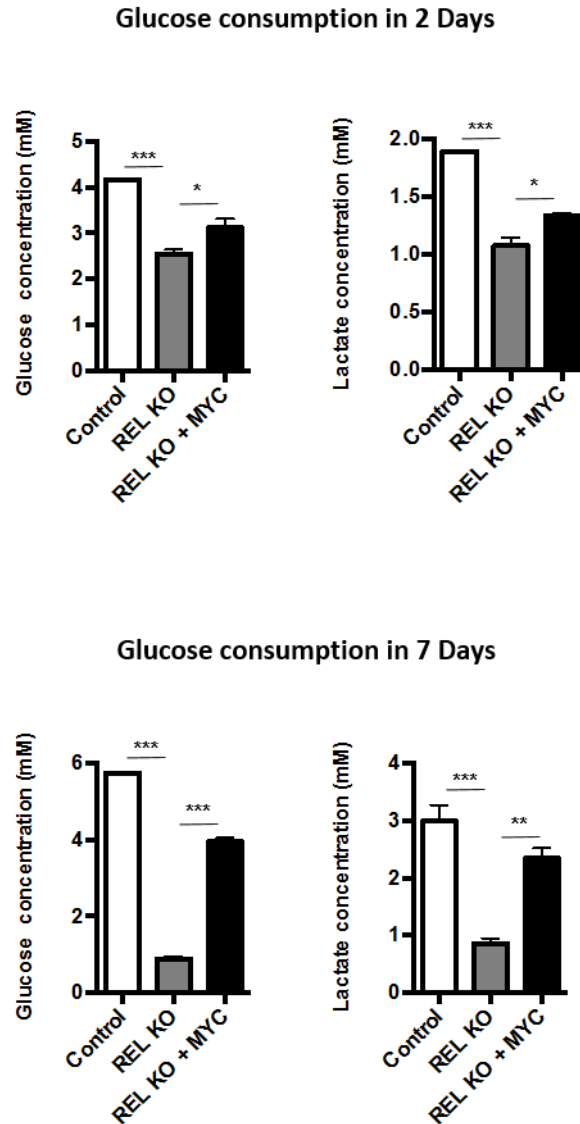
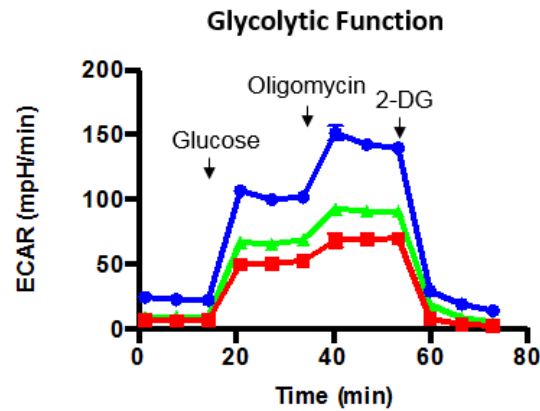


FIGURE 19. c-Rel KO cells show reduced glucose consumption

sgRNA control, c-Rel KO, and c-Rel KO cells transduced with c-Myc were cultured under 2% FBS for 2 and 7 days. Glucose consumptions were calculated by subtracting end glucose concentration from the initial glucose concentration. For all panels, values represent mean \pm SEM of pooled samples of equal numbers of cells from 3 clones (n=3; *p<0.05, **p<0.01, ***p<0.001; one-way ANOVA).

A.



B.

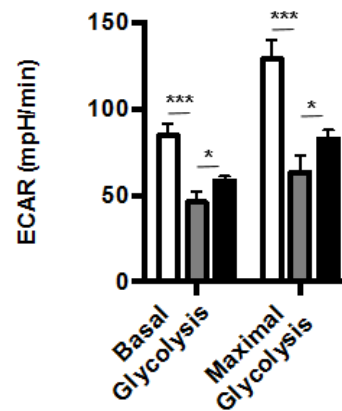
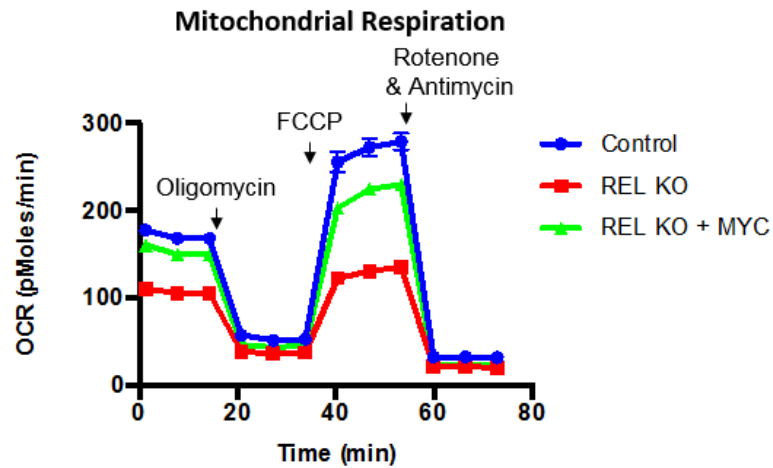


FIGURE 20. c-Rel KO Jurkat cells exhibit impaired glycolysis that can be partially reversed by c-Myc overexpression

The glycolytic activity and maximum glycolytic capacity were measured using a Seahorse extracellular flux analyzer. A) sgRNA control Jurkat cells transduced with GFP, c-Rel KO cells transduced with GFP, and c-Rel KO cells transduced with c-Myc and GFP were starved under 2% FBS for 1 day and glucose starved for 2 hours, then treated sequentially with D-glucose, oligomycin, and 2-Deoxyglucose (2-DG) while the extracellular acidification rates (ECAR) were measured in real-time. B) Basal glycolysis is measured by the glycolytic rate defined by ECAR following the addition of glucose. Maximum glycolytic capacity is represented by ECAR following oligomycin addition. Values represent mean \pm SEM of pooled samples of equal numbers of cells from 3 clones (n=6; *p<0.05, ***p<0.001; one-way ANOVA).

A.



B.

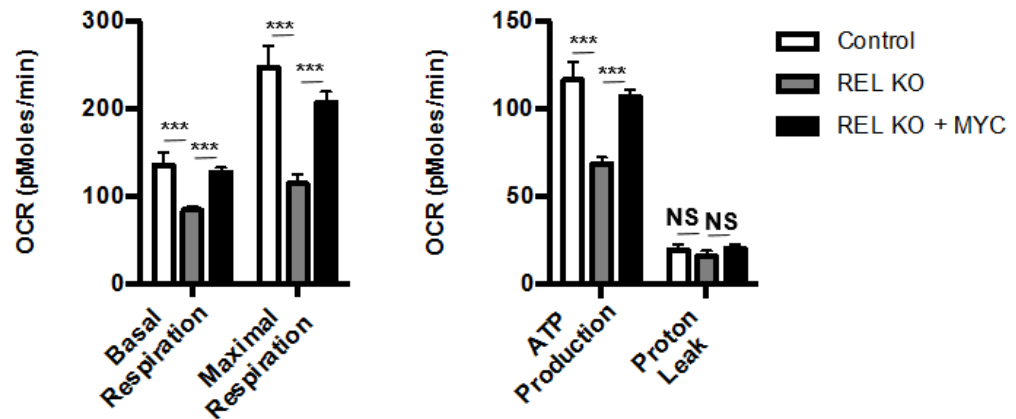


FIGURE 21. c-Rel KO Jurkat cells show compromised mitochondrial respiration that can be rescued by c-Myc

sgRNA control Jurkat cells transduced with GFP, c-Rel KO cells transduced with GFP, and c-Rel KO cells transduced with c-Myc and GFP were starved under 2% FBS for 1 day followed by the Seahorse mitochondrial stress test. A) Real-time recording of oxygen consumption rate (OCR). B) Quantitation of the four mitochondrial parameters from the same assay. Values represent mean \pm SEM of pooled samples of equal numbers of cells from 3 clones (n=6; ***p<0.001; one-way ANOVA).

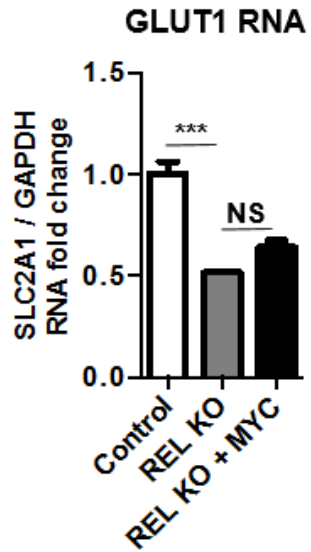
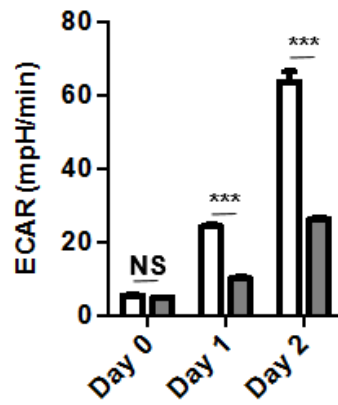


FIGURE 22. c-Myc does not fully rescue GLUT1 deficiency in c-Rel KO cells

sgRNA control Jurkat cells(Ctrl) transduced with GFP, c-Rel KO cells transduced with GFP, and c-Rel KO cells transduced with c-Myc and GFP were starved under 2% FBS for 2 days followed by RT-qPCR quantification of GLUT1 mRNA. Data were normalized with GAPDH. Values represent mean \pm SEM of pooled samples of equal amounts of RNA from 3 clones (n=3; ***p<0.001; one-way ANOVA).

A.



B.

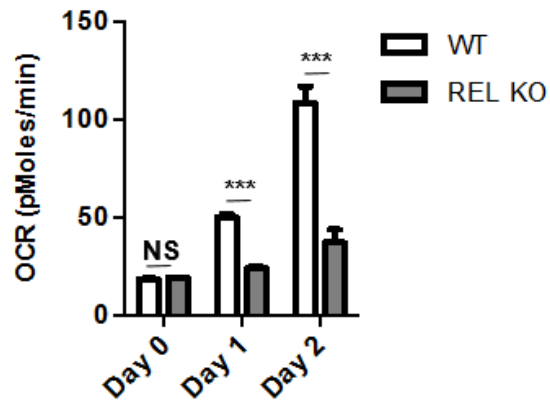


FIGURE 23. Primary CD4⁺ T cells isolated from c-Rel KO mice show significant defects in both mitochondrial respiration and glycolysis

CD4⁺ T cells isolated from WT and c-Rel KO mice were stimulated with anti-CD3 and anti-CD28 antibodies for up to two days followed by the Seahorse glycolysis stress (A) and mitochondrial stress (B) tests. Real-time recording of the extracellular acidification rates (ECAR) and the oxygen consumption rate (OCR) is shown in the upper and lower panel, respectively. Values represent mean \pm SEM of pooled samples of equal numbers of cells from 2 mice (** $p < 0.001$; Student's t-test).

CHAPTER 4: Discussion and Future Directions

Discussion:

Results reported here indicate that c-Rel is a critical positive regulator of cellular metabolism. c-Rel deficient Jurkat T cells completely lost their proliferation capacity under nutrient-limited conditions, thereby establishing its role as an indispensable stress responder. It has previously been shown that the proliferation defect of c-Rel knockout mouse T cells is caused by a lack of IL-2 production upon TCR activation (Liou et al., 1999). However, our data demonstrated that in humans c-Rel also controls intrinsic ability of these cells to survive and proliferate under nutrient stress independent of IL-2 signaling. Notably, many phenotypes of the c-Rel KO Jurkat cells are opposite to those observed in mouse T cells deficient in Hif-1 α , another previously-identified metabolic regulator. Mouse T cells purified from conditional Hif-1 α KO mice exhibit increased proliferation and cytokine production, including IL-2 and IFN- γ (Korn et al., 2009; McNamee et al., 2013). Whereas c-Rel KO Jurkat cells share many similarities with conditional c-Myc KO mouse T cells, such as compromised glycolysis and glutaminolysis as well as reduced cell growth and proliferation (Wang et al., 2011), c-Rel and c-Myc are not complete substitutes for one another. For example, our data showed that glucose transporter 1 (GLUT1), a key regulator of glycolysis that controls glucose import, is significantly downregulated in the c-Rel KO cells. However, this decrease was only partially reversed by c-Myc

overexpression (Fig. 22). Yet, as demonstrated by others, ectopically expressed c-Myc can directly induce the expression of GLUT1 (Osthus et al., 2000; Dang et al., 2009). Similarly, c-Myc overexpression in c-Rel KO cells almost fully rescued the defect in mitochondrial respiration but only partially reversed the glycolysis defect, which is also consistent with the incomplete rescue of GLUT1 expression.

These findings indicate that T cell metabolism is maintained by multiple positive and negative regulators that could not compensate for each other; deficiency in any one of them leads to serious functional defects. Our data show that c-Myc only accounts for a portion of c-Rel's downstream effects, and that c-Rel also controls other metabolic effectors independent of c-Myc. A number of studies have suggested that c-Myc can work as a global transcriptional amplifier (Nie et al., 2012; Lin et al., 2012). Thus, based on our results we propose the following model (Fig. 24): Upon nutrient sensor-mediated activation, c-Rel activates many target genes while upregulating c-Myc expression. c-Myc, in turn, enhances the expression of many of these genes, i.e., their common targets. In other words, c-Rel upregulates c-Myc to amplify many of its own targets. Without the presence of c-Rel, c-Myc alone is insufficient to boost the expression of these genes to required levels, resulting in only a partial rescue. Thus, c-Rel and c-Myc work in a synergistic manner to drive metabolic reprogramming and promote cell proliferation.

The similar metabolic reprogramming observed during T cell activation and cancer transformation, i.e., the Warburg effect, and the fact that Jurkat is a human

acute T-cell leukemia line provides a strong implication of a mechanistic role of c-Rel in tumor development: By boosting glycolysis and mitochondrial respiration, c-Rel may provide cancer cells with the metabolic support needed to overcome the tumor-driven nutrient deprivation in the microenvironment. In addition to metabolic reprogramming, there are other ways c-Rel could contribute to cancer development. It has been shown in mouse that c-Rel directly controls expression of numerous inflammatory cytokines, such as IL-1 β , IL-6, TNF- α and IFN- γ (Pahl et al., 1999), many of which are frequently dysregulated in autoimmune diseases and cancer. In fact, c-Rel-deficient mice are resistant to autoimmune diseases such as arthritis, experimental autoimmune encephalomyelitis, colitis, and type 1 diabetes (Campbell et al., 2000; Hilliard et al., 2002; Gilmore and Gerondakis, 2011). Knowing the long-recognized link between inflammation and cancer, it would not be surprising if c-Rel contributes to tumorigenesis through promotion of inflammation.

The reliance of cancer growth on altered metabolic pathways such as aerobic glycolysis, glutaminolysis, and fatty acid synthesis provides the basis for attacking cancer cells through metabolic interference. Targeting cancer metabolism has therefore become an area of intense interest and research focus. There have been some promising evidence on the efficacy of this approach (Galluzzi et al., 2013; Bensaad et al., 2013; Zhao et al., 2013; Morales et al., 2015; Marini et al., 2016). Given the critical importance of c-Rel in driving metabolic reprogramming to facilitate cell growth and proliferation under nutrient-deprived conditions, c-Rel could be a promising target for novel metabolism-based treatment strategies. Indeed, one study

has shown that integrin $\beta 3$ -expressing human pancreatic carcinoma cells are resistant to EGFR tyrosine kinase inhibitor erlotinib and nutrient deprivation. However, this resistance can be overcome through inhibition of c-Rel using RNAi, and a combination of c-Rel shRNA and erlotinib successfully blocked tumor cell growth (Seguin et al., 2014). Therefore, similar approaches that interfere with glycolysis through c-Rel inhibition represent a potentially important avenue for developing novel combination cancer therapies.

Future directions:

This study showed for the first time in a human system that c-Rel is a key metabolic regulator that promotes glycolysis and mitochondrial respiration in T cells. In the absence of c-Rel, Jurkat cells simply could not survive serum starvation. It also showed that nutrient stress-evoked induction of c-Myc is critically dependent on c-Rel. Nevertheless, there are important questions remain to be answered. First, although this study revealed an interesting connection between metabolic regulation and tumor growth under nutrient stress, the significance of this connection to the broader biological context *in vivo* remains uncertain. Further *in vivo* studies will be needed to assess the impact of c-Rel knockout or knockdown under physiological or pathological conditions. Second, since c-Myc only accounts for part of c-Rel's metabolic effect, more thorough investigation are needed to identify other direct or indirect c-Rel targets that account for the remainder of the c-Rel-regulated metabolic

pathway. Third, because cancer and activated effector T cells share many similar metabolic characteristics, drugs that directly targeting key metabolic regulators such as c-Rel will likely interfere with metabolism of both cell types. Thus, in order to achieve desired targeting effect, it is crucial to understand the similarities and differences of metabolic pathways in cancer and in normal T cells. Due to the complexity and scale of interconnection among metabolic networks, extensive research in these pathways remains to be done before their therapeutic potential can be realized.

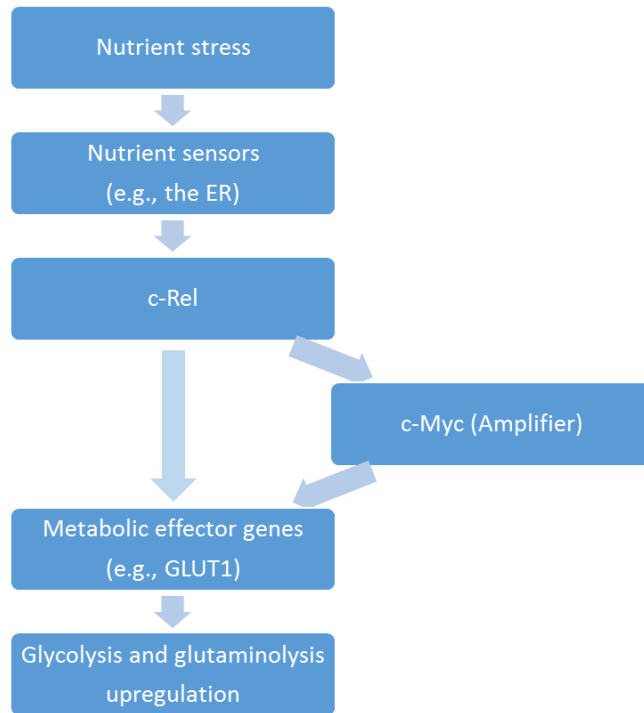


FIGURE 24. Proposed mechanism for c-Rel-regulated metabolic reprogramming

Nutrient sensors detect stress signals and activates c-Rel through the NF- κ B pathway. c-Rel in turn transactivates its target genes while upregulating c-Myc expression. Enhanced c-Myc activity then further amplify metabolism-related genes activated by c-Rel, leading to increased glycolysis and glutaminolysis.

CHAPTER 5: Materials and Methods

Cell lines

Jurkat cells were obtained from ATCC and grown in RPMI 1640 supplemented with 10% or 2% fetal bovine serum (FBS, Hyclone), 2mM L-Glutamine (Gibco), and penicillin/streptomycin (Sigma).

Animals

C57BL/6 (B6) mice that carry a c-Rel gene null mutation were previously generated and backcrossed to B6 mice for 12 generations before used in this study (Hilliard et al., 2002; Liou et al., 1999). B6 control mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). All mice were housed in the University of Pennsylvania animal care facilities under pathogen-free conditions and all procedures were pre-approved by the institutional animal care and use committee.

Plasmid vectors

pCW-Cas9 and pLX-sgRNA were a gift from Eric Lander & David Sabatini (Addgene plasmid # 50661 & 50662). Lentiviral vectors used for c-Rel rescue and c-Myc overexpression were constructed based on the FUGW vector from the David Baltimore lab. pGL4.12[luc2CP] luciferase reporter vector was purchased from Promega (Madison, Wisconsin, USA).

Plasmid construction

pLX-sgRNA vectors

sgRNA sequences were ligated to U6 promoter sequence and sgRNA scaffold sequence using overlap-extension PCR, followed by restriction and ligation using the XhoI and NheI restriction site in the pLX-sgRNA plasmid. The sgRNA sequences used to generate the final c-Rel KO clones are summarized in the following table:

sgRNA sequence	Location (from 5')	Strand
AAAACGCATTCCCCTCTGCC	52 bp	Reverse
TAGAGATAATTGAACAACCC	45 bp	Forward
TAATTGAACAACCCAGGCAG	51 bp	Forward

c-Rel and c-Myc rescue vectors

The backbone vector was first create by inserting an internal ribosome entry site (IRES) element and the EGFP coding sequence into the FUGW vector. Human c-Rel and c-Myc cDNA was then cloned into the vector using the following primer sets:

	Forward primer	Reverse primer
c-Rel	ATGGCCTCCGGTGCGTATAACCCG	TTATACTTGAAAAAATTCATATGGAAAG GAG
c-Myc	CTGGATTTTTTTTCGGGTAGTGGA AAACC	TTACGCACAAGAGTTCCGTAGCTG

c-Myc promoter reporter vector

A 2kb genomic fragment containing the 5' UTR and the proximal promoter of the c-Myc gene were amplified by PCR from human genomic DNA, and subcloned into the HindIII/NcoI sites of the pGL4.12[luc2P] luciferase reporter plasmid using the following primer set:

Forward primer	Reverse primer
GGCCAAGCTTAAAAGGCACGGAAGTAATA CTCCTCTCCTC	TCTTCCATGGTCTAAGCAGCTGCAAGG AGAGCCTTTC

Lentivirus production and cell transduction

Lentiviruses used for generating the c-Rel KO, c-Rel KO c-Rel rescue, c-Rel KO c-Myc rescue, and the control cells were produced by co-transfecting the respective vectors with the 3rd generation packaging system into 80% confluent 293T cells using the FuGENE 6 (Promega, Madison, WI) transfection reagent according to the manufacturer's instructions. Virus-containing supernatants were collected and filtered 36-48 h after transfection, and were either used immediately or stored at -80°C.

Jurkat cells were transduced in viral supernatants containing 10µg/mL polybrene at a density of 2×10^5 cells/mL, and were centrifuged under 3000 rpm, 30°C for 90 min, followed by replacement of fresh culture medium.

Generation of c-Rel KO cells using the CRISPR/Cas9 system (Fig. 25)

c-Rel KO Jurkat cells were generated using the CRISPR/Cas9 system described by Wang et al. Specifically, 8 candidate sgRNA target sequences were generated based on complementarity to the N-terminus region of the c-Rel coding sequence. These target sequences were then fused to the sgRNA scaffold sequence and cloned into the pLX-sgRNA vector using the XhoI and NheI restriction sites. The resulting vectors were used to produce lentiviruses in 293T cells by co-transfection with the 3rd generation packaging system. Jurkat cells were first transduced by lentivirus produced from the doxycycline-inducible Cas9 vector (pCW-Cas9) and selected under 1.5µg/mL puromycin for 3 days. The resulting cells were expanded and transduced by the c-Rel-targeting pLX-sgRNA lentiviruses and subsequently selected under 6µg/mL blasticidin in the presence of 750ng/mL doxycycline for one week. The cells were then expanded without doxycycline until sufficient number is obtained. c-Rel protein levels were then analyzed by western blotting.

Clonal selection to establish pure c-Rel KO lines

Jurkat cells were serially diluted to 0.5 cells / 200µL / well in 96-well plates and grew until density reaches confluency. The cells were then transferred to 12-well plates and expanded until sufficient number is obtained. c-Rel protein levels were then analyzed by western blotting.

PMA/Ionomycin stimulation of Jurkat cells

1x10⁶/mL Jurkat cells were stimulated in standard culture medium containing 10% FBS with the addition of 10ng/mL PMA and 1μM ionomycin for 4 hours before analyzed.

Mouse T cell isolation and stimulation

CD4⁺ T cells were isolated from spleens of 8-12 week old, age-matched male and female c-Rel KO and B6 mice using surface antigen and magnetic beads-based negative selection kit (mouse CD4⁺ T Cell Isolation Kit, Miltenyi Biotec) according to the manufacturer's instructions. After isolation, the WT or c-Rel KO cells were expanded with a 1:3 (cell:bead) ratio with mouse anti-CD3/CD28 beads for 2 days.

Western blotting

Cells were lysed in SDS and total protein concentration determined. 30μg protein was loaded to each lane, and separated by SDS-PAGE. After transferring to a nitrocellulose membrane, it was blocked with 5% milk in TBST and probed with the following primary antibodies, overnight at 4°C: c-Rel (#4727, rabbit polyclonal, 1:2000, Cell signaling), c-Myc (#9402, rabbit monoclonal, 1:1000, Cell Signaling), β-Actin (1:10000, mouse monoclonal, Sigma). HRP-conjugated secondary antibodies (anti-mouse or anti-rabbit IgG, 1:1000, GE healthcare) were incubated at room temperature for 2 hours. Detection was done using Amersham enhanced chemiluminescence (ECL).

Cell sorting by flow cytometry

Flow cytometric sorting was performed on Jurkat cells transduced with vectors carrying a GFP marker. Cells were analyzed on a BD FACS Aria II sorter. Data were analyzed with the FlowJo software.

Quantitative RT-PCR

Total RNA was extracted with RNEasy (Qiagen, Valencia, CA) according to the manufacturer's instructions. Reverse transcription was performed with oligo dT primers. Real-time PCR was carried out in an Applied Biosystems 7500 system with Power SYBR Green PCR Master Mix (Applied Biosystems). Relative levels of gene expression were determined with GAPDH as the control. The primers used in the experiments are summarized in Table 1.

Gene	Forward primer	Reverse primer
GAPDH	GAGTCAACGGATTTGGTCGT	TTGATTTTGGAGGGATCTCG
IL-2	GTCACAAACAGTGCACCTAC	CCCTGGGTCTTAAGTGAAAG
c-Rel	GCAGGAATCAATCCATTCAA	CAGGAGGAAGAGCAGTCGTC
c-Myc	TTCGGGTAGTGGAACCAG	AGTAGAAATACGGCTGCACC
SLC2A1	AAAGTGACAAGACACCCGAG	TGTCAGGTTTGGAAGTCTCATC
NR4A2	TCCTCGAAAACGCCTGTAAC	TGGAGTTAAGAAATCGGAGCTG
NR4A3	AGTGTCTCAGTGTTGGAATGG	AGGAGAAGGTGGAGAGGG

SLC27A2	CGAGAAAAGTTGGTGCTGTTG	TGGGAACCTCTGACGCAATATC
PPARGC1A	ACCAAACCCACAGAGAACAG	GGTCAGAGGAAGAGATAAAGTTG
TFAM	GATTGAAGTTGGACGAAAGGATC	CGATAGCTTCAGGTATTGAGACC

Luciferase reporter assay

Control and c-Rel KO Jurkat cells were transfected with either promoterless control plasmid or pGL4.12[luc2CP] c-Myc reporter plasmid by electroporation using a Gene Pulser II electroporator (Bio-Rad) at a concentration of 20µg/5x10⁶ cells/mL. Cells were rested for 4 hours and stimulated with 10ng/ml PMA and 1µM ionomycin. 12 hours after stimulation, cells were lysed, and luciferase activities were measured using a luciferase assay kit (Promega) and a GloMax 20/20 luminometer (Promega), according to the manufacturer's protocols.

Seahorse metabolic analysis

For Jurkat cells, cells were moved to RPMI 1640 medium containing 2% FBS for 1 day before seeded to 96-well format plates. For mouse primary T cells, cells were expanded with a 1:3 (cell:bead) ratio with mouse anti-CD3/CD28 beads for up to 2 days before seeding. Cells were harvested and resuspended at 5x10⁶ cells/mL in appropriate Seahorse media. 40µL of cells were added to 140µL of appropriate seahorse media and plated in XF96 cell culture microplates that were precoated with

25µL of Cell tak reagent overnight according to manufacturer's instructions (Corning, 354240).

For examining glycolysis, the Seahorse glyco-stress test kit was used with additions of the following reagents to cells in glucose-free Seahorse media: 10mM glucose, 1µM oligomycin and 50mM 2-DG. To determine the rate of glycolysis, cells were incubated in glucose-free medium and the increased ECAR response upon stimulation of glycolysis by glucose and oligomycin was determined. Oligomycin inhibits mitochondrial ATP synthase (complex V) forcing glycolysis to compensate for the lack of ATP production in oxidative phosphorylation. Finally, 2-Dexoglucose (2DG) was added to inhibit glycolysis. Glycolysis was calculated as the difference between the ECAR after addition of glucose and the baseline ECAR. Glycolytic capacity was calculated as the difference between the ECAR after addition of oligomycin and the baseline ECAR.

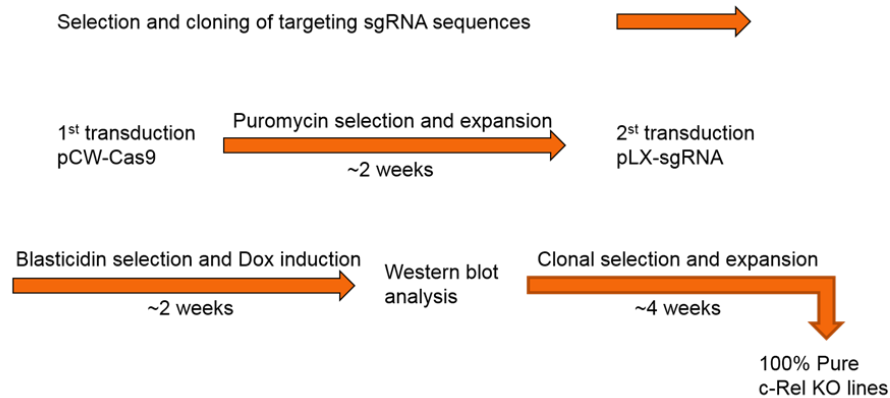
For examining mitochondrial function, a mito-stress kit was used with additions of the following reagents to cells in appropriate Seahorse media: 1µM Oligomycin, 1µM fluoro-carbonyl cyanide phenylhydrazine (FCCP, an uncoupler of respiration and oxidative ATP synthesis), and pre-mixed 0.5 µM rotenone + antimycin A solution (Electron Transport Chain complex I and III inhibitors). Maximum respiratory capacity was calculated as the difference between the Oxygen Consumption Rate (OCR) after addition of FCCP and the OCR after addition of rotenone/antimycin A. Spare respiratory capacity was calculated as the difference

between maximal (post FCCP) and basal respiration. Plates were run on a Seahorse XF96 Extracellular Flux Analyzer according to the manufacturer's protocols.

Statistical analyses

All statistics were performed using Prism 5 (GraphPad, La Jolla, CA). Data were expressed as mean \pm standard error of mean (SEM) throughout the manuscript. For comparisons between two groups, two-tailed Student's t-test was used for evaluation of statistical significance. For comparisons across multiple groups, one-way ANOVA with Bonferroni post-test adjustment was used. Data shown are representative of two to three independent experiments. NS, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

A.



B.

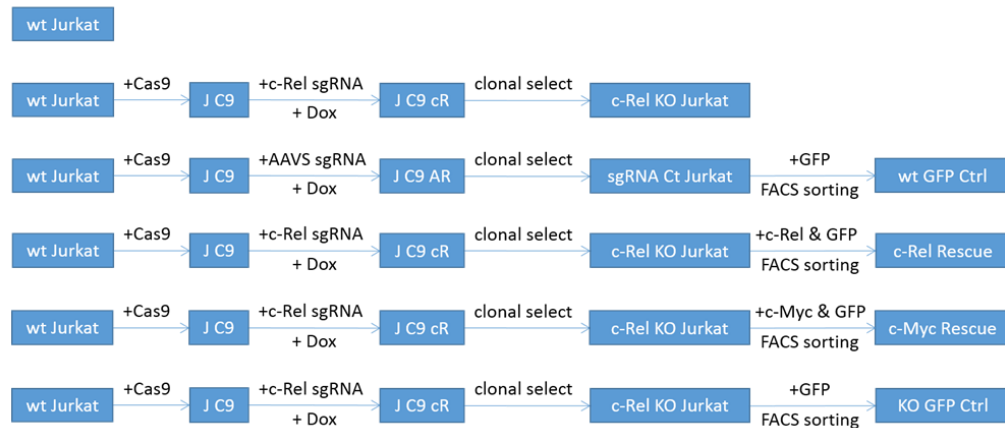


FIGURE 25. Process of generating c-Rel knockout Jurkat cells and control cells

A) Jurkat cells were first transduced by tet-on Cas9-expressing lentiviral vector carrying a puromycin selection marker and treated with puromycin for 2 weeks. Selected cells were then transduced with a sgRNA-expression lentiviral vector carrying a blasticidin selection marker and treated with blasticidin and doxycycline for 2 weeks. The resulting cells were analyzed by western blotting. sgRNA groups showing strong c-Rel knockdown were continued with clonal selection to produce pure knockout lines.

B) sgRNA control cell underwent the same processes as the c-Rel KO cells, except with a different sgRNA. Control groups used to assess rescuing effect of c-Rel and c-Myc overexpression underwent the same number of transductions and selections, except with a GFP only lentiviral vector.

REFERENCES

- Adhikary S, Eilers M. Transcriptional regulation and transformation by Myc proteins. *Nat Rev Mol Cell Biol.* 2005; 6:635–645.
- Altman BJ, Dang CV. Normal and cancer cell metabolism: lymphocytes and lymphoma. *FEBS J* 2012;279(15):2598–2609.
- Antonsson A, Hughes K, Edin S, Grundström T. Regulation of c-Rel nuclear localization by binding of Ca²⁺/calmodulin. *Mol Cell Biol.* 2003;23:1418-27.
- Arkan M, et al. IKKb links inflammation to obesity-induced insulin resistance. *Nat. Med.* 2005;11, 191–198
- Arpaia N, Campbell C, Fan X, et al. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature* 2013;504(7480):451–455.
- Baker RG, et al. NF-κB, inflammation, and metabolic disease. *Cell Metab.* 2011;5, 11–22
- Barbi J, Pardoll D, Pan F. Metabolic control of the Treg/Th17 axis. *Immunol Rev* 2013;252(1):52–77.
- Barth TF, Martin-Subero JL, Joos S, et al. Gains of 2p involving the REL locus correlate with nuclear c-Rel protein accumulation in neoplastic cells of classical Hodgkin's lymphoma. *Blood.* 2003;101:3681-86.
- Barton GM. A calculated response: control of inflammation by the innate immune system. *J. Clin. Invest.* 2008;118, 413–420
- Bensaad K, Harris AL. Cancer metabolism as a therapeutic target: metabolic synthetic lethality. *Oncology* 2013;27(467):473
- Ben-Shoshan J, Maysel-Auslender S, Mor A, et al. Hypoxia controls CD4⁺CD25⁺ regulatory T-cell homeostasis via hypoxia-inducible factor-1α. *Eur J Immunol* 2008;38(9):2412–2418.
- Boffa DJ, Feng B, Sharma V, et al. Selective loss of c-Rel compromises dendritic cell activation of T lymphocytes. *Cell Immunol.* 2003;222:105-15.
- Bollinger T, Gies S, Naujoks J, et al. HIF-1α- and hypoxia-dependent immune responses in human CD4⁺CD25^{high} T cells and T helper 17 cells. *J Leukoc Biol* 2014;96(2):305–312.

- Bonneville M, Fournie JJ. Sensing cell stress and transformation through Vgamma9Vdelta2 T cell-mediated recognition of the isoprenoid pathway metabolites. *Microbes Infect* 2005;7(3):503–509.
- Bradford JW, Baldwin AS IKK/nuclear factor- κ B and oncogenesis: roles in tumor-initiating cells and in the tumor microenvironment. *Adv Cancer Res* 2014;121: 125–145.
- Brownell, E., B. Mathieson, H.A. Young, J. Keller, J.N. Ihle, and N.R. Rice. Detection of c-rel-related transcripts in mouse hematopoietic tissues, fractionated lymphocyte populations, and cell lines. *Molecular & Cellular Biology* 1987;7:1304-1309.
- Bruzzese L, Fromonot J, By Y, et al. NF-kappaB enhances hypoxia-driven T-cell immunosuppression via upregulation of adenosine A2A receptors. *Cell Signal* 2014;26(5):1060–1067.
- Bunting K, Rao S, Hardy K, et al. Genome-wide analysis of gene expression in T cells to identify targets of the NF- κ B transcription factor c-Rel. *J Immunol.* 2007;178:7097-109.
- Burkitt MD, Hanedi AF, Duckworth CA, Williams JM, Tang JM, O'Reilly LA, Putoczki TL, Gerondakis S, Dimaline R, Caamano JH, Pritchard DM. NF- κ B1, NF- κ B2 and c-Rel differentially regulate susceptibility to colitis-associated adenoma development in C57BL/6 mice. *J Pathol* 2015;236(3):326–336.
- Burkitt MD, Williams JM, Duckworth CA, O'Hara A, Hanedi A, Varro A, Caamano JH, Pritchard DM. Signaling mediated by the NF- κ B sub-units NF- κ B1, NF- κ B2 and c-Rel differentially regulate *Helicobacter felis*-induced gastric carcinogenesis in C57BL/6 mice. *Oncogene* 2013;32(50):5563–5573.
- Buttgereit F, Burmester GR, Brand MD. Bioenergetics of immune functions: fundamental and therapeutic aspects. *Immunol Today* 2000;21(4):192–199.
- Cai D, et al. Local and systemic insulin resistance resulting from hepatic activation of IKKb and NF- κ B. *Nat. Med.* 2005;11, 183–190
- Caldwell CC, Kojima H, Lukashev D, et al. Differential effects of physiologically relevant hypoxic conditions on T lymphocyte development and effector functions. *J Immunol* 2001;167(11):6140–6149.
- Campbell IK, Gerondakis S, O'Donnell K, Wicks IP. Distinct roles for the NF- κ B1 (p50) and c-Rel transcription factors in inflammatory arthritis. *J Clin Invest.* 2000;105:1799-806.

- Carmody RJ, Ruan Q, Liou HC, Chen YH. Essential roles of c-Rel in Toll-like receptor-induced interleukin-23 p19 gene expression in dendritic cells. *Journal of Immunology* 2007;178:186-191.
- Carrasco D, Weih F, Bravo R. Developmental expression of the mouse c-rel proto-oncogene in hematopoietic organs. *Development*. 1994;120:2991-3004.
- Chaban Y, Boekema EJ, Dudkina NV. Structures of mitochondrial oxidative phosphorylation supercomplexes and mechanisms for their stabilisation. *Biochim Biophys Acta* 2014 Apr;1837(4):418–426.
- Chang CH, Curtis JD, Maggi LB, Jr., et al. Posttranscriptional control of T cell effector function by aerobic glycolysis. *Cell* 2013;153(6):1239–1251.
- Chen C, Edelstein LC, G  linas C. The Rel/NF-  B family directly activates expression of the apoptosis inhibitor Bcl-xL. *Mol Cell Biol*. 2000;20:2687-95.
- Chen E, Hrdlickov   R, Nehyba J, Longo DL, Bose Jr HR, Li C-CH. Degradation of proto-oncoprotein c-Rel by the ubiquitin-proteasome pathway. *J Biol Chem*. 1998;273:35201-7.
- Chen E, Li CC. Association of Cdk2/cyclin E and NF-  B complexes at G1/S phase. *Biochem Biophys Res Commun*. 1998;249:728-34.
- Chiang SH, et al. The protein kinase IKKe regulates energy balance in obese mice. *Cell* 2009;138, 961–975
- Chien YH, Zeng X, Prinz I. The natural and the inducible: interleukin (IL)-17-producing gammadelta T cells. *Trend Immunol* 2013;34(4):151–154.
- Cho JH, Brant SR. Recent insights into the genetics of inflammatory bowel disease. *Gastroenterology*. 2011;140:1704-12.
- Choy E. Understanding the dynamics: pathways involved in the pathogenesis of rheumatoid arthritis. *Rheumatology* 2012;51(Suppl 5):v3–v11.
- Cobbold SP. The mTOR pathway and integrating immune regulation. *Immunology* 2013;140(4):391–398.
- Coller HA, Grandori C, Tamayo P, Colbert T, Lander ES, Eisenman RN, et al. Expression analysis with oligonucleotide microarrays reveals that MYC regulates genes involved in growth, cell cycle, signaling, and adhesion. *Proc Natl Acad Sci U S A*. 2000; 97:3260–3265.

- Courtine E, Pène F, Cagnard N, et al. Critical role of cRel subunit of NF- κ B in sepsis survival. *Infect Immun*. 2011;79:1848-54.
- Courtois G, Gilmore TD. Mutations in the NF- κ B signaling pathway: implications for human disease. *Oncogene*. 2006;25:6831-43.
- Cramer T, Yamanishi Y, Clausen BE, et al. HIF-1 α is essential for myeloid cell-mediated inflammation. *Cell*. 2003;112(5):645–657.
- Crespo J, Sun H, Welling TH, et al. T cell anergy, exhaustion, senescence, and stemness in the tumor microenvironment. *Curr Opin Immunol* 2013;25(2):214–221.
- Currie E, Schulze A, Zechner R, Walther TC, Farese RV Jr. Cellular fatty acid metabolism and cancer. *Cell Metab* 2013;18:153–161
- Curry CV, Ewton AA, Olsen RJ, et al. Prognostic impact of C-REL expression in diffuse large B-cell lymphoma. *J Hematop*. 2009;2:20-6.
- Dang CV, Le A, Gao P. MYC-induced cancer cell energy metabolism and therapeutic opportunities. *Clin Cancer Res*. 2009; 15:6479–6483.
- Dang CV, Resar LMS, Emison E, Kim S, Li Q, Prescott JE, Wonsey D, Zeller K. Function of the c-Myc oncogenic transcription factor. *Exp. Cell Res*. 1999;253:63–77.
- Dang CV. Rethinking the Warburg effect with Myc micromanaging glutamine metabolism. *Cancer Res*. 2010; 70:859–862.
- Dang EV, Barbi J, Yang HY, et al. Control of T(H)17/T(reg) balance by hypoxia-inducible factor 1. *Cell* 2011;146(5):772–784.
- Deberardinis RJ, Sayed N, Ditsworth D, Thompson CB. Brick by brick: metabolism and tumor cell growth. *Curr Opin Genet Dev*. 2008; 18:54–61.
- Di Donato JA, et al. NF- κ B and the link between inflammation and cancer. *Immunol. Rev*. 2012;246, 379–400
- Du JW, Xu KY, Fang LY, Qi XL. Interleukin-17, produced by lymphocytes, promotes tumor growth and angiogenesis in a mouse model of breast cancer. *Mol Med Rep* 2012;6(5):1099–1102.
- Edinger AL. Controlling cell growth and survival through regulated nutrient transporter expression. *Biochem J* 2007;406(1):1–12.
- Eilers M, Eisenman RN. Myc's broad reach. *Genes Dev*. 2008; 22:2755–2766.

- Enciso-Mora V, Broderick P, Ma Y, et al. A genome-wide association study of Hodgkin's lymphoma identifies new susceptibility loci at 2p16.1 (REL), 8q24.21 and 10p14 (GATA3). *Nat Genet.* 2010;42:1126-30.
- Eyre S, Hinks A, Flynn E, et al. Confirmation of association of the REL locus with rheumatoid arthritis susceptibility in the UK population. *Ann Rheum Dis.* 2010;69:1572-3.
- Fan Y, Rayet B, G  linas C. Divergent C-terminal transactivation domains of Rel/NF-  B proteins are critical determinants of their oncogenic potential in lymphocytes. *Oncogene.* 2004;23:1030-42.
- Fernandez PC, Frank SR, Wang L, Schroeder M, Liu S, Greene J, et al. Genomic targets of the human c-Myc protein. *Genes Dev.* 2003; 17:1115  1129.
- Firestein GS. Evolving concepts of rheumatoid arthritis. *Nature* 2003;423(6937):356  361.
- Fogal V, Richardson AD, Karmali PP, Scheffler IE, Smith JW, Ruoslahti E. Mitochondrial p32 protein is a critical regulator of tumor metabolism via maintenance of oxidative phosphorylation. *Mol Cell Biol* 2010;30:1303  1318
- Fox CJ, Hammerman PS, Thompson CB. Fuel feeds function: energy metabolism and the T-cell response. *Nat Rev Immunol* 2005;5(11):844  852.
- Frauwirth KA, Riley JL, Harris MH, et al. The CD28 signaling pathway regulates glucose metabolism. *Immunity* 2002;16(6):769  777.
- Freie BW, Eisenman RN. Ratcheting Myc. *Cancer Cell.* 2008; 14:425  426.
- Fukuhara N, Tagawa H, Kameoka Y, et al. Characterization of target genes at the 2p15-16 amplicon in diffuse large B-cell lymphoma. *Cancer Sci.* 2006;97:499-504.
- Gaber T, Dziurla R, Tripmacher R, et al. Hypoxia inducible factor (HIF) in rheumatology: low O2! See what HIF can do! *Ann Rheum Dis* 2005;64(7):971  980.
- Gaber T, Haupl T, Sandig G, et al. Adaptation of human CD4+T cells to pathophysiological hypoxia: a transcriptome analysis. *J Rheumatol* 2009;36(12):2655  2669.
- Gaber T, Strehl C, Sawitzki B, Hoff P, Buttgereit F. Cellular energy metabolism in T-lymphocytes. *Int Rev Immunol.* 2015;34(1):34-49.

- Galluzzi L, Kepp O, Vander Heiden MG, Kroemer G. Metabolic targets for cancer therapy. *Nat Rev Drug Discov* 2013;12:829–846
- Gao P, Tchernyshyov I, Chang TC, Lee YS, Kita K, Ochi T, Zeller KI, De Marzo AM, Van Eyk JE, Mendell JT, Dang CV. c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. *Nature*. 2009;458(7239):762-5.
- Geginat J, Paroni M, Facciotti F, et al. The CD4-centered universe of human T cell subsets. *Semin Immunol* 2013;25(4):252–262.
- Geginat J, Sallusto F, Lanzavecchia A. Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD4(+) T cells. *J Exp Med* 2001;194(12):1711–1719.
- Gerondakis S, Seibenlist U. Roles of the NF- κ B pathway in lymphocyte development and function. *Cold Spring Harb Perspect Biol*. 2010;2:a000182
- Gerriets VA, Rathmell JC. Metabolic pathways in T cell fate and function. *Trends Immunol* 2012;33(4):168–173.
- Gieling RG, Eisharkawy AM, Caamaño JH, et al. The c-Rel subunit of nuclear factor κ B regulates murine liver inflammation, woundhealing, and hepatocyte proliferation. *Hepatology*. 2010;51:922-31.
- Gilmore TD, Cormier D, Jean-Jacques J, Gapuzan M-E. Malignant transformation of primary chicken spleen cells by human transcription factor c-Rel. *Oncogene*. 2001;20:7098-103.
- Gilmore TD, Gerondakis S. The c-Rel transcription factor in development and disease. *Genes Cancer* 2011;2(7): 695–711.
- Gilmore TD. Introduction to NF- κ B: players, pathways, perspectives. *Oncogene*. 2006;25:6680-4.
- Gilmore TD. Multiple mutations contribute to the oncogenicity of the retroviral oncoprotein v-Rel. *Oncogene*. 1999;18:6925-37.
- Gnanaprakasam JN, Wang R. MYC in Regulating Immunity: Metabolism and Beyond. *Genes (Basel)*. 2017;8(3). pii: E88.
- Gordan JD, Thompson CB, Simon MC. HIF and c-Myc: sibling rivals for control of cancer cell metabolism and proliferation. *Cancer Cell*. 2007; 12:108–113.

- Gregersen PK, Amos CI, Lee AT, et al. REL, encoding a member of the NF- κ B family of transcription factors, is a newly defined risk locus for rheumatoid arthritis. *Nat Genet.* 2009;41:820-3.
- Grigoriadis G, Vasanthakumar A, Banerjee A, Grumont R, Overall S, Gleeson P, Shannon F, Gerondakis S. c-Rel controls multiple discrete steps in the thymic development of Foxp3⁺ CD4 regulatory T cells. *PLoS One.* 2011;6(10):e26851.
- Grivennikov SI, et al. Immunity, inflammation, and cancer. *Cell* 2010;140, 883–899
- Grumont RJ, Gerondakis S. Rel induces interferon regulatory factor 4 (IRF-4) expression in lymphocytes: modulation of interferonregulated gene expression by rel/nuclear factor κ B. *J Exp Med.* 2000;191:1281-92.
- Grumont RJ, Hochrein H, O’Keeffe M, et al. c-Rel regulates interleukin 12 p70 expression in CD8(+) dendritic cells by specifically inducing p35 gene transcription. *J Exp Med.* 2001;194:1021-32.
- Grumont RJ, Rourke IJ, O’Reilly LA, et al. B lymphocytes differentially use the Rel and NF- κ B1 transcription factors to regulate cell cycle progression and apoptosis in quiescent and mitogen-activated cells. *J Exp Med.* 1998;187:663-74.
- Guo QM, Malek RL, Kim S, Chiao C, He M, Ruffly M, et al. Identification of c-myc responsive genes using rat cDNA microarray. *Cancer Res.* 2000; 60:5922–5928.
- Guppy M, Greiner E, Brand K. The role of the Crabtree effect and an endogenous fuel in the energy metabolism of resting and proliferating thymocytes. *Eur J Biochem* 1993;212(1):95–99.
- Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell.* 2011; 144:646–674.
- Hann SR, Eisenman RN. Proteins encoded by the human c-myc oncogene: differential expression in neoplastic cells. *Mol Cell Biol.* 1984; 4:2486–2497.
- Hardie DG. AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nat Rev Mol Cell Biol* 2007;8(10):774–785.
- Hartmann S, Gesk S, Scholtysik R, et al. High resolution SNP array genomic profiling of peripheral T cell lymphomas, not otherwise specified, identifies a subgroup with chromosomal aberrations affecting the REL locus. *Br J Haematol.* 2010;148:402-12.

- Hayden MS, Ghosh S. Shared principles in NF- κ B signaling. *Cell* 2008;132, 344–362
- Heckman CA, Mehew JW, Boxer LM. NF- κ B activates Bcl-2 expression in t(14;18) lymphoma cells. *Oncogene*. 2002;21:3898-908.
- Herbel C, Patsoukis N, Bardhan K, Seth P, Weaver JD, Boussiotis VA. Clinical significance of T cell metabolic reprogramming in cancer. *Clin Transl Med*. 2016;5(1):29.
- Hilliard BA, Mason N, Xu L, Sun J, Lamhamedi-Cherradi SE, Liou HC, Hunter C, Chen YH. Critical Roles of c-Rel in Autoimmune Inflammation and Helper T Cell Differentiation. *Journal of Clinical Investigation* 2002;110:843-850.
- Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 2010;363(8):711–23.
- Hori S. c-Rel: a pioneer in directing regulatory T-cell lineage commitment? *Eur J Immunol*. 2010;40:664-7.
- Hotamisligil GS and Erbay E. Nutrient sensing and inflammation in metabolic diseases. *Nat. Rev. Immunol*. 2008;8, 923–934
- Hotamisligil GS. Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. *Cell* 2010;140, 900–917
- Houldsworth J, Mathew S, Rao PH, et al. REL proto-oncogene is frequently amplified in extranodal diffuse large cell lymphoma. *Blood*. 1996;87:25-9.
- Houldsworth J, Olshen AB, Cattoretti G, et al. Relationship between REL amplification, REL function, and clinical and biologic features in diffuse large B-cell lymphoma. *Blood*. 2004;103:1862-8.
- Hsu PP, Sabatini DM. Cancer cell metabolism: Warburg and beyond. *Cell*. 2008;134:703–707.
- Huang D, Chen Y, Ruetsche M, Phelps CB, Ghosh G. X-ray crystal structure of proto-oncogene product c-Rel bound to the CD28 response element of IL-2. *Structure*. 2001;9:669-78.
- Huguet, C., F. Bouali, P.J. Enrietto, D. Stehelin, B. Vandenbunder, and C. Abbadie. The avian transcription factor c-Rel is expressed in lymphocyte precursor cells and antigen-presenting cells during thymus development. *Developmental Immunology* 1998;5:247-261.

- Hunter JE, Leslie J, Perkins ND. c-Rel and its many roles in cancer: an old story with new twists. *Br J Cancer*. 2016;114(1):1-6.
- Ikejiri A, Nagai S, Goda N, et al. Dynamic regulation of Th17 differentiation by oxygen concentrations. *Int Immunol* 2012;24(3):137–146.
- Ishikawa H, Asano M, Kanda T, Gélinas C, Ito Y. Two novel functions associated with the Rel oncoproteins: DNA replication and cell-specific transcriptional activation. *Oncogene*. 1993;8:2889-96.
- Isomura I, Palmer S, Grumont RJ, et al. c-Rel is required for the development of thymic Foxp3⁺ CD4 regulatory T cells. *J Exp Med*. 2009;206:3001-14.
- Janse M, Lamberts LE, Franke L, et al. Three ulcerative colitis susceptibility loci are associated with primary sclerosing cholangitis and indicate a role for IL2, REL, and CARD9. *Hepatology*. 2011;53:1977-85.
- Jerby L, Ruppin E. Predicting drug-targets and biomarkers of cancer via genome-scale metabolic modeling. *Clin Cancer Res*. 2012; 18 xx-xx.
- John S, Weiss JN, Ribalet B. Subcellular localization of hexokinases I and II directs the metabolic fate of glucose. *PloS one*. 2011;6(3):e17674.
- Johnson RF, et al. p53-dependent regulation of mitochondrial energy production by the RelA subunit of NF- κ B. *Cancer Res*. 2011;71, 5588–5597
- Johnson RF, Perkins ND. Nuclear factor- κ B, p53, and mitochondria: regulation of cellular metabolism and the Warburg effect. *Trends Biochem Sci*. 2012;37(8):317-24.
- Joos S, Granzow M, Holtgreve-Grez H, et al. Hodgkin's lymphoma cell lines are characterized by frequent aberrations on chromosomes 2p and 9p including REL and JAK2. *Int J Cancer*. 2003;103:489-95.
- Joos S, Ota?o-Joos MI, Ziegler S, et al. Primary mediastinal (thymic) B-cell lymphoma is characterized by gains of chromosomal material including 9p and amplification of the REL gene. *Blood*. 1996;87:1571-8.
- Kaadige MR, Elgort MG, Ayer DE. Coordination of glucose and glutamine utilization by an expanded Myc network. *Transcription*. 2010; 1:36–40.
- Karin M. NF- κ B as a critical link between inflammation and cancer. *Cold Spring Harb Perspect Biol* 2009;1(5): a000141

- Kasznicki J, Sliwinska A, Drzewoski J. Metformin in cancer prevention and therapy. *Ann Transl Med* 2014;2:57
- Kawai T and Akira S. TLR signaling. *Semin. Immunol.* 2007;19, 24–32
- Kawauchi K, et al. p53 regulates glucose metabolism through an IKK- NF- κ B pathway and inhibits cell transformation. *Nat. Cell Biol.* 2008;10, 611–618
- Kim J, Woo AJ, Chu J, Snow JW, Fujiwara Y, Kim CG, et al. A Myc network accounts for similarities between embryonic stem and cancer cell transcription programs. *Cell.* 2010; 143:313–324.
- Kim JW, Dang CV. Cancer's molecular sweet tooth and the Warburg effect. *Cancer Res.* 2006; 66:8927–8930.
- Kim JW, Gao P, Liu YC, Semenza GL, Dang CV. Hypoxia-inducible factor 1 and dysregulated c-Myc cooperatively induce vascular endothelial growth factor and metabolic switches hexokinase 2 and pyruvate dehydrogenase kinase 1. *Mol Cell Biol.* 2007; 27:7381–7393.
- Kim JW, Tchernyshyov I, Semenza GL, Dang CV. HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. *Cell Metab.* 2006; 3:177–185.
- Kim JW, Zeller KI, Wang Y, et al. Evaluation of myc E-box phylogenetic footprints in glycolytic genes by chromatin immunoprecipitation assays. *Mol Cell Biol.* 2004;24:5923–36.
- Kontgen, F., R.J. Grumont, A. Strasser, D. Metcalf, R. Li, D. Tarlinton, and S. Gerondakis. Mice lacking the c-rel proto-oncogene exhibit defects in lymphocyte proliferation, humoral immunity, and interleukin-2 expression. *Genes & Development* 1995;9:1965-1977.
- Koppenol WH, Bounds PL, Dang CV. Otto Warburg's contributions to current concepts of cancer metabolism. *Nat Rev Cancer.* 2011; 11:325–337.
- Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 cells. *Annu Rev Immunol* 2009;27:485–517.
- Kunsch C, Ruben SM, Rosen CA. Selection of optimal κ B/Rel DNAbinding motifs: interaction of both subunits of NF- κ B with DNA is required for transcriptional activation. *Mol Cell Biol.* 1992;12:4412-21.
- Lago R, et al. Leptin beyond body weight regulation – current concepts concerning its role in immune function and inflammation. *Cell. Immunol.* 2008;252, 139–145

- Lamhamedi-Cherradi SE, Zheng S, Hilliard BA, Xu L, Sun J, Alsheadat S, Liou HC, Chen YH. Transcriptional regulation of type I diabetes by NF-kappa B. *Journal of Immunology* 2003;171:4886-4892.
- Leeman JR, Weniger MA, Barth TF, Gilmore TD. Deletion analysis and alternative splicing define a transactivation inhibitory domain in human oncoprotein REL. *Oncogene*. 2008;27:6770-81.
- Levens DL. Disentangling the MYC web. *Proc Natl Acad Sci USA*. 2002;99(9):5757-9.
- Levens DL. Reconstructing MYC. *Genes Dev*. 2003;17(9):1071-7.
- Li F, Wang Y, Zeller KI, Potter JJ, Wonsey DR, O'Donnell KA, et al. Myc stimulates nuclearly encoded mitochondrial genes and mitochondrial biogenesis. *Mol Cell Biol*. 2005; 25:6225–6234.
- Lim HY, Ho QS, Low J, Choolani M, Wong KP. Respiratory competent mitochondria in human ovarian and peritoneal cancer. *Mitochondrion*. 2011;11:437–443.
- Lin CY, Lovén J, Rahl PB, Paranal RM, Burge CB, Bradner JE, Lee TI, Young RA. Transcriptional amplification in tumor cells with elevated c-Myc. *Cell*. 2012;151(1):56-67.
- Liou HC, Hsia CY. Distinctions between c-Rel and other NF-κB proteins in immunity and disease. *BioEssays*. 2003;25:767-80.
- Liou HC, Jin Z, Tumang J, Andjelic S, Smith KA, Liou ML. c-Rel is crucial for lymphocyte proliferation but dispensable for T cell effector function. *Int Immunol*. 1999;11(3):361-71.
- Liou H-C, Smith KA. The roles of c-Rel and interleukin-2 in tolerance: a molecular explanation of self-nonself discrimination. *Immunol Cell Biol*. 2011;89:27-32.
- Long M, Park SG, Strickland I, Hayden MS, Ghosh S. Nuclear factor κB modulates regulatory T cell development by directly regulating expression of Foxp3 transcription factor. *Immunity*. 2009;31:921-31.
- Lu D, Thompson JD, Gorski GK, Rice NR, Mayer MG, Yunis JJ. Alterations at the rel locus in human lymphoma. *Oncogene*. 1991;6:1235-41.
- Maciolek JA, Alex Pasternak J, Wilson HL. Metabolism of activated T lymphocytes. *Curr Opin Immunol* 2014;27C:60–74.

- MacIver NJ, Michalek RD, Rathmell JC. Metabolic regulation of T lymphocytes. *Annu Rev Immunol* 2013;31:259–283.
- Mader A, Bruderlein S, Wegener S, et al. U-HO1, a new cell line derived from a primary refractory classical Hodgkin lymphoma. *Cytogenet Genome Res.* 2007;119:204-10.
- Marini C, Bianchi G, Buschiazzo A, Ravera S, Martella R, Bottoni G, Petretto A et al. Divergent targets of glycolysis and oxidative phosphorylation result in additive effects of metformin and starvation in colon and breast cancer. *Sci Rep* 2016;6:19569
- Martin AG, Fresno M. Tumor necrosis factor- α activation of NF- κ B requires the phosphorylation of Ser-471 in the transactivation domain of c-Rel. *J Biol Chem.* 2000;275:24383-91.
- Martin AG, San-Antonio B, Fresno M. Regulation of nuclear factor κ B transactivation: implication of phosphatidylinositol 3-kinase and protein kinase C ζ in c-Rel activation by tumor necrosis factor α . *J Biol Chem.* 2001;276:15840-9.
- Martin-Subero JI, Gesk S, Harder L, et al. Recurrent involvement of REL and BCL11A loci in classical Hodgkin lymphoma. *Blood.* 2002;99:1474-7.
- Mason NJ, Liou H-C, Hunter CA. T-cell intrinsic expression of c-Rel regulates Th1 responses essential for resistance to *Toxoplasma gondii*. *J Immunol.* 2004;172:3704-11.
- Mauro C, et al. NF- κ B controls energy homeostasis and metabolic adaptation by upregulating mitochondrial respiration. *Nat. Cell Biol.* 2011;13, 1272–1279
- McNamee EN, Korn Johnson D, Homann D, Clambey ET. Hypoxia and hypoxia-inducible factors as regulators of T cell development, differentiation, and function. *Immunol Res.* 2013;55(1-3):58-70.
- Medzhitov R. Origin and physiological roles of inflammation. *Nature* 2008;454, 428–435
- Menssen A, Hermeking H. Characterization of the c-MYC-regulated transcriptome by SAGE: identification and analysis of c-MYC target genes. *Proc Natl Acad Sci USA.* 2002; 99:6274–6279.
- Meyer N, Penn LZ. Reflecting on 25 years with MYC. *Nat Rev Cancer.* 2008; 8:976–990.

- Michalek RD, Gerriets VA, Jacobs SR, et al. Cutting edge: distinct glycolytic and lipid oxidative metabolic programs are essential for effector and regulatory CD4⁺ T cell subsets. *J Immunol* 2011;186(6):3299–3303.
- Miller DM, Thomas SD, Islam A, Muench D, Sedoris K. c-Myc and cancer metabolism. *Clin Cancer Res*. 2012;18(20):5546-53
- Molon B, Cali B, Viola A. T Cells and Cancer: How Metabolism Shapes Immunity. *Front Immunol*. 2016;7:20.
- Morales DR, Morris AD. Metformin in cancer treatment and prevention. *Annu Rev Med* 2015;66:17–29
- Morrish F, Neretti N, Sedivy JM, Hockenbery DM. The oncogene c-Myc coordinates regulation of metabolic networks to enable rapid cell cycle entry. *Cell Cycle*. 2008; 7:1054–1066.
- Natoli G, Sacconi S, Bosisio D, Marazzi I. Interactions of NF-kappaB with chromatin: the art of being at the right place at the right time. *Nature Immunology* 2005;6:439-445.
- Nie Z, Hu G, Wei G, Cui K, Yamane A, Resch W, Wang R, Green DR, Tessarollo L, Casellas R, Zhao K, Levens D. c-Myc is a universal amplifier of expressed genes in lymphocytes and embryonic stem cells. *Cell*. 2012;151(1):68-79.
- Numasaki M, Fukushi J, Ono M, et al. Interleukin-17 promotes angiogenesis and tumor growth. *Blood* 2003;101(7):2620–2627.
- Ohta A, Diwanji R, Kini R, Subramanian M, et al. In vivo T cell activation in lymphoid tissues is inhibited in the oxygen-poor microenvironment. *Front Immunol* 2011;2:27.
- Ohta A, Gorelik E, Prasad SJ, et al. A2A adenosine receptor protects tumors from antitumor T cells. *Proc Natl Acad Sci USA* 2006;103(35):13132–13137.
- Ohta A, Kini R, Subramanian M, et al. The development and immunosuppressive functions of CD4(+) CD25(+) FoxP3(+) regulatory T cells are under influence of the adenosine-A2A adenosine receptor pathway. *Front Immunol* 2012;3:190.
- Osthus RC, Shim H, Kim S, Li Q, Reddy R, Mukherjee M, Xu Y, Wonsey D, Lee LA, Dang CV. Dereglulation of glucose transporter 1 and glycolytic gene expression by c-Myc. *J Biol Chem*. 2000 Jul 21;275(29):21797-800.

- Pahl HL. Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene* 1999;18:6853-6866.
- Palomero T, Lim WK, Odom DT, Sulis ML, Real PJ, Margolin A, et al. NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. *Proc Natl Acad Sci U S A*. 2006; 103:18261–18266.
- Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* 2012;12(4):252-64.
- Pelengaris S, Khan M, Evan G. c-MYC: more than just a matter of life and death. *Nat Rev Cancer*. 2002; 2:764–776.
- Perkins ND. Integrating cell-signalling pathways with NF-kB and IKK function. *Nat. Rev. Mol. Cell Biol.* 2007;8, 49–62
- Perkins ND. The diverse and complex roles of NF-kB subunits in cancer. *Nat. Rev. Cancer* 2012;12, 121–132
- Pico de Coaña Y, Choudhury A, Kiessling R. Checkpoint blockade for cancer therapy: revitalizing a suppressed immune system. *Trends Mol Med* 2015;21(8):482-91.
- Powell JD, Delgoffe GM. The mammalian target of rapamycin: linking T cell differentiation, function, and metabolism. *Immunity* 2010;33(3):301–311.
- Rahman M, Hasan MR. Cancer metabolism and drug resistance. *Metabolites* 2015;5:571–600
- Ramos JC, Ruiz Jr P, Ratner L, Reis IM, Brites C, Pedroso C, Byrne Jr GE, Toomey NL, Andela V, Harhaj EW, Lossos IS, Harrington Jr WJ. IRF-4 and c-Rel expression in antiviral-resistant adult T-cell leukemia/lymphoma. *Blood* 2007;109(7): 3060–3068.
- Rao S, Gerondakis S, Woltring D, Shannon MF. c-Rel is required for chromatin remodeling across the IL-2 gene promoter. *J Immunol.* 2003;170:3724-31.
- Reader JC, Zhao XF, Butler MS, Rapoport AP, Ning Y. REL-positive double minute chromosomes in follicular lymphoma. *Leukemia*. 2006;20:1624-6.
- Riley JL. PD-1 signaling in primary T cells. *Immunol Rev* 2009;229(1):114–125.

- Ruan Q, Kameswaran V, Tone Y, Li L, Liou HC, Greene MI, Tone M, Chen YH. Development of Foxp3(+) regulatory T cells is driven by the c-Rel enhanceosome. *Immunity* 2009;31:932-940.
- Ruan Q, Kameswaran V, Zheng S, Wang J, Liou HC, Beg AA, Chen YH. The Th17 Immune Response Is Controlled By A c-Rel-ROR γ -ROR γ T Transcriptional Axis. *Immunity* 2010;31:932-940.
- Saibil SD, Jones RG, Deenick EK, et al. CD4+ and CD8+ T cell survival is regulated differentially by protein kinase C θ , c-Rel and protein kinase B. *J Immunol.* 2007;178:2932-9.
- Salani B, Marini C, Rio AD, Ravera S, Massollo M, Orengo AM, Amaro A et al. Metformin impairs glucose consumption and survival in Calu-1 cells by direct inhibition of hexokinase-II. *Sci Rep* 2013;3:2070
- Sander S, Bullinger L, Wirth T. Repressing the repressor: a new mode of MYC action in lymphomagenesis. *Cell Cycle.* 2009; 8:556–559.
- Sanjabi S, Williams KJ, Sacconi S, Zhou L, Hoffmann A, Ghosh G, Gerondakis S, Natoli G, Smale ST. A c-Rel subdomain responsible for enhanced DNA-binding affinity and selective gene activation. *Genes Dev* 2005;19(18):2138–2151.
- Sattar N, McCarey DW, Capell H, McInnes IB. Explaining how “high-grade” systemic inflammation accelerates vascular risk in rheumatoid arthritis. *Circulation* 2003;108(24):2957–2963.
- Schodel J, Oikonomopoulos S, et al. High-resolution genome-wide mapping of HIF-binding sites by ChIP-seq. *Blood* 2011;117(23):e207–e217.
- Scholz CC, Taylor CT. Hydroxylase-dependent regulation of the NF-kappaB pathway. *Biol Chem* 2013;394(4):479–493.
- Schreiber J, Jenner RG, Murray HL, Gerber GK, Gifford DK, Young RA. Coordinated binding of NF- κ B family members in the response of cells to lipopolysaccharide. *Proc Natl Acad Sci USA.* 2006;103:5899-904.
- Scott DA, Richardson AD, Filipp FV, Knutzen CA, Chiang GG, Ronai ZA, Osterman AL, Smith JW. Comparative metabolic flux profiling of melanoma cell lines: beyond the Warburg effect. *J Biol Chem.* 2011;286:42626–42634.
- Seguin L, Cheresch DA1 et al. An integrin β 3-KRAS-RalB complex drives tumour stemness and resistance to EGFR inhibition. *Nat Cell Biol.* 2014;16(5):457-68.

- Semenza GL. Hypoxia-inducible factor 1 (HIF-1) pathway. *Sci STKE* 2007;2007(407):cm8.
- Semenza GL. Hypoxia-inducible factors in physiology and medicine. *Cell* 2012;148(3):399–408.
- Semenza GL. Oxygen sensing, hypoxia-inducible factors, and disease pathophysiology. *Annu Rev Pathol* 2014;9:47–71.
- Shaw RJ, Kosmatka M, Bardeesy N, et al. The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. *Proc Natl Acad Sci USA* 2004;101(10):3329–3335.
- Shi LZ, Wang R, Huang G, et al. HIF1 α -dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells. *J Exp Med* 2011;208(7):1367–1376.
- Shim H, Dolde C, Lewis BC, Wu CS, Dang G, Jungmann RA, et al. c-Myc transactivation of LDH-A: implications for tumor metabolism and growth. *Proc Natl Acad Sci U S A*. 1997;94:6658–6663.
- Solinas G and Karin M. JNK1 and IKK β : molecular links between obesity and metabolic dysfunction. *FASEB J*. 2010;24, 2596–2611
- Son JS, Sahoo A, Chae CS, Hwang JS, Park ZY, Im SH. JunB and c-Rel cooperatively enhance Foxp3 expression during induced regulatory T cell differentiation. *Biochem Biophys Res Commun*. 2011;407:141–7.
- Starczynowski DT, Reynolds JG, Gilmore TD. Deletion of either C-terminal transactivation subdomain enhances the in vitro transforming activity of human transcription factor REL in chicken spleen cells. *Oncogene*. 2003;22:6928–36.
- Starczynowski DT, Trautmann H, Pott C, et al. Mutation of an IKK phosphorylation site within the transactivation domain of REL in two patients with human B-cell lymphoma enhances REL's in vitro transforming activity. *Oncogene*. 2007;26:2685–94.
- Staudt LM. Oncogenic activation of NF- κ B. *Cold Spring Harb Perspect Biol*. 2010;2:a000109.
- Strange A, Capon F, Spencer CC, et al. A genome-wide association study identifies new psoriasis susceptibility loci and an interaction between HLA-c and ERAP1. *Nat Genet*. 2010;42:985–90.

- Strehl C, Fangradt M, Fearon U, et al. Hypoxia: how does the monocyte-macrophage system respond to changes in oxygen availability? *J Leukoc Biol* 2014;95(2):233–241.
- Tandon P, Gallo CA, Khatri S, et al. Requirement for ribosomal protein S6 kinase 1 to mediate glycolysis and apoptosis resistance induced by Pten deficiency. *Proc Natl Acad Sci USA* 2011;108(6):2361–2365.
- Teicher BA, Linehan WM, Helman LJ. Targeting cancer metabolism. *Clin Cancer Res.* 2012; 18.
- Tenoever BR, et al. Multiple functions of the IKK-related kinase IKKe in interferon-mediated antiviral immunity. *Science* 2007;315, 1274–1278
- Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* 2012;366(26):2443–54.
- Tornatore L, Thotakura AK, Bennett J, Moretti M, Franzoso G. The nuclear factor kappa B signaling pathway: integrating metabolism with inflammation. *Trends Cell Biol.* 2012;22(11):557–66.
- Trynka G, Zhernakova A, Romanos J, et al. Coeliac disease–associated risk variants in TNFAIP3 and REL implicate altered NF- κ B signaling. *Gut.* 2009;1078–83.
- Vallejo AN, Weyand CM, Goronzy JJ. T-cell senescence: a culprit of immune abnormalities in chronic inflammation and persistent infection. *TrendsMolMed* 2004;10(3):119–124.
- van der Windt GJ, Everts B, Chang CH, et al. Mitochondrial respiratory capacity is a critical regulator of CD8⁺ T cellmemory development. *Immunity* 2012;36(1):68–78.
- van der Windt GJ, Pearce EL. Metabolic switching and fuel choice during T-cell differentiation and memory development. *Immunol Rev* 2012;249(1):27–42.
- van Essen D, Zhu Y, Sacconi S. A feed-forward circuit controlling inducible NF-kappaB target gene activation by promoter histone demethylation. *Mol Cell* 2010;39(5): 750–760.
- Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science.* 2009;324(5930):1029–33.

- Vang KB, Yang J, Pagan AJ, et al. CD28 and c-Rel-dependent pathways initiate regulatory T cell development. *J Immunol*. 2010;184:4074-7.
- Varadé J, Palomino-Morales R, Ortego-Centeno N, et al. Analysis of the REL polymorphism rs13031237 in autoimmune diseases. *Ann Rheum Dis*. 2011;70:711-2.
- Verbist KC, Wang R, Green DR. T cell metabolism and the immune response. *Semin Immunol* 2012;24(6):399–404.
- Voet D, Voet JG. *Biochemistry*. 4th ed. Weinheim: Wiley-VCH; 2011.
- Wang H, Flach H, Onizawa M, et al. Negative regulation of Hif1a expression and TH17 differentiation by the hypoxia-regulated microRNA miR-210. *Nat Immunol* 2014;15(4):393–401.
- Wang R, Dillon CP, Shi LZ, Milasta S, Carter R, Finkelstein D, McCormick LL, Fitzgerald P, Chi H, Munger J, Green DR. The transcription factor Myc controls metabolic reprogramming upon T lymphocyte activation. *Immunity*. 2011;35:871–882
- Wang T, Wei JJ, Sabatini DM, Lander ES. *Science*. Genetic screens in human cells using the CRISPR-Cas9 system. 2014;343(6166):80-4.
- Wang Y, Rickman BH, Poutahidis T, Schlieper K, Jackson EA, Erdman SE, Fox JG, Horwitz BH. c-Rel is essential for the development of innate and T cell-induced colitis. *J Immunol* 2008;180(12): 8118–8125.
- Warburg O. Origin of cancer cells. *Oncologia* 1956;9(2):75–83.
- Ward JP. Oxygen sensors in context. *Biochim Biophys Acta* 2008;1777(1):1–14.
- Weaver CT, Hatton RD, Mangan PR, Harrington LE. IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu Rev Immunol* 2007;25:821–852.
- Weinberg F, Hamanaka R, Wheaton WW, Weinberg S, Joseph J, Lopez M, Kalyanaraman B et al. Mitochondrial metabolism and ROS generation are essential for Kras-mediated tumorigenicity. *Proc Natl Acad Sci USA* 2010;107:8788–8793
- Weniger MA, Gesk S, Ehrlich S, et al. Gains of REL in primary mediastinal B-cell lymphoma coincide with nuclear accumulation of REL protein. *Genes Chromosomes Cancer*. 2007;46:406-15.

- Wise DR, DeBerardinis RJ, Mancuso A, Sayed N, Zhang XY, Pfeiffer HK, Nissim I, Daikhin E, Yudkoff M, McMahon SB, Thompson CB. Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction. *Proc Natl Acad Sci U S A*. 2008;105(48):18782-7.
- Wunderlich FT, et al. Interleukin-6 signaling in liver parenchymal cells suppresses hepatic inflammation and improves systemic insulin action. *Cell Metab*. 2010;12, 237–249
- Zeller KI, Jegga AG, Aronow BJ, O'Donnell KA, Dang CV. An integrated database of genes responsive to the Myc oncogenic transcription factor: identification of direct genomic targets. *Genome Biol*. 2003; 4:R69.
- Zeller KI, Zhao X, Lee CW, Chiu KP, Yao F, Yustein JT, et al. Global mapping of c-Myc binding sites and target gene networks in human B cells. *Proc Natl Acad Sci USA*. 2006; 103:17834–17839.
- Zhang X, et al. Hypothalamic IKK β /NF- κ B and ER stress link overnutrition to energy imbalance and obesity. *Cell* 2008;135, 61–73
- Zhao Y, Butler EB, Tan M. Targeting cellular metabolism to improve cancer therapeutics. *Cell Death Dis* 2013;4:e532
- Zheng Y, Vig M, Lyons J, Van Parijs L, Beg AA. Combined deficiency of p50 and cRel in CD4⁺ T cells reveals an essential requirement for nuclear factor κ B in regulating mature T cell survival and in vivo function. *J Exp Med*. 2003 197:861-74.